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         Micro-Metastases

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13. ABSTRACT *(Maximum 200 words)*

This project is studying the development of dendritic cell vaccines for immune therapy of breast cancer. We have developed techniques for isolation and immunogen loading of murine splenic dendritic cells which induced humoral and cellullar immunity in a model system (BSA). Translation to a tumor antigen (CEA) produced weak immune responses and antitumor effects. This system requires large amounts of immunogen to be effective. We have developed a means to induce CEA expression in dendritic cells using a gene transfer vector (replication defective recombinant adenovirus [AdCEA]). Ex vivo transfection of splenic dendritic cells with AdCEA followed by i.v. infection induced moderate anti-CEA antibody responses without detectable cellular immune responses or antitumor effects. Direct injection of the free AdCEA i.v. or i.p. produced marked anti-CEA antibody responses but also without detectable cellular immune responses or antitumor effects. In the context of cutaneous polynucleotide immunization by gene gun, co-delivery of GM-CSF cDNA significantly augments CEA-specific humoral and cellular immune responses. Further studies of cytokine and co-stimulatory molecule cDNA co-delivery are underway to augment the immune response to polynucleotide immunization via gene gun or i.m. injection.

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Albert J Banfio, MD   _9/5/96_
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Department of the Army
Annual Report
Grant No. DAMD 17-94-J-4402

Introduction:

The underlying reason for failure to cure patients diagnosed with breast cancer is the presence of micro-metastases in approximately one-third of breast cancer patients.[1,2] The stimulation of antitumor immune responses represents one of the most effective ways to treat low tumor burdens that are microscopically or clinically occult.[3,4] The objective of our proposal is to determine whether novel dendritic cell (DC) vaccines containing a human tumor-associated antigen (carcinoembryonic antigen) can induce systemic immunity and eradication of micro-metastases in a syngeneic murine breast cancer model which expresses human CEA. The feasibility and efficacy of this CEA vaccine strategy would be applicable to other putative tumor-specific or associated tumor antigens.

Body:

Immunization with dendritic cells pulsed with antigenic protein ex vivo

The first year of this proposal has consisted of the examination and manipulation of dendritic cells for the purpose of developing a potent, novel tumor vaccine that will be applicable to human clinical trials. Dendritic cells are highly potent antigen-presenting cells (APCs) able to initiate and stimulate cellular immune response *in vitro* and *in vivo*.[5] Initiation of immune response to an antigen clearly includes the role of APCs responsible for intracellular antigen processing with subsequent cell-contact mediated stimulation of naive T cells.[6] Of the several types of APCs (macrophages, monocytes, activated B cells, etc.), dendritic cells have been shown to be the most potent or effective with estimates of 40 to 100-fold increments in activity over macrophages or B cells.[7,8] Dendritic cells appear operative in initiation of immune responses to transplantation antigens, soluble proteins, viral infections, etc.[9] They are capable of activity in both primary and secondary immune responses with a large body of *in vitro* studies and increasing observations *in vivo*.[8,10] In this regard, tumor vaccine studies employing autologous tumor cells transduced with genes encoding a variety of cytokines demonstrated the greatest immunoprotection and therapy using GM-CSF (a dendritic cell growth factor).

Furthermore, techniques currently exist for isolation and *in vitro* culture of human dendritic cells so that strategies of dendritic cell vaccines can be readily translated to human trials. We have become adept at isolating dendritic cells from mouse spleens using the procedure of Steinman et al.[10,11] Briefly, spleens are collagenase-digested and passed through a cell strainer. The dendritic cells are then floated over a dense bovine albumin gradient and selected by nonadherence to plastic tissue culture plates after overnight culture. To examine loading of dendritic cells with soluble protein antigens as a means of immunization, we needed to devise a dendritic cell enrichment procedure which avoids exposure to heterologous proteins such as bovine albumin and fetal calf serum. We successfully modified the enrichment procedure of Steinman et al.[10,11] to eliminate protein exposure except of the specific protein being studied by floating dendritic cells over a Percoll gradient (instead of BSA) and use of rat serum in the overnight culture instead of fetal calf serum.

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Dendritic cells are equipped with cell surface molecules which enable their role in triggering T helper and cytolytic T cell responses. Highly purified dendritic cells have a rich display of both class I and II major histocompatibility complex (MHC) molecules[6,12] as well as a potent array of co-stimulatory molecules including LFA-3, ICAM-1 and B7/BB1.[13] These cell surface molecules stimulate naive mononuclear cells from other murine strains to proliferate in the mixed lymphocyte reaction. We have verified the quality of our dendritic cell preparations using this assay and have found them to be 100-fold more potent than whole splenic mononuclear cells.

Dendritic cells exposed to soluble protein antigens can mediate T cell immune responses both *in vitro* and *in vivo*. Dendritic cells (prior to prolonged culture) can take up soluble proteins, process them and induce T cell responses *in vitro* reflecting immune T cell receptor interaction with class II MHC-peptide complexes.[7] *In vivo* studies have also shown that such *in vitro* "pulsed" dendritic cells can induce immune T cell and antibody responses.[10,11]

In seeking to exploit these properties of dendritic cells to develop a vaccination strategy, we initially examined the ability of protein-pulsed dendritic cells administered by various routes and at varying doses to elicit humoral and cellular immune responses. We decided to do these initial studies using immune response to bovine serum albumin (BSA) given the need for large amounts of pure protein for such studies.

Table 1. Anti-BSA Antibody Response Elicited by BSA-Pulsed Dendritic Cells
Delivered by Various Routes[a]

Route	Group 1		Group 2	
	Day 28	Day 35	Day 56	Day 63
Intraperitoneal	450 ± 200[b]	13,000 ± 6,000	3,300 ± 1,000	110,000 ± 50,000
Intravenous	1,200 ± 200	160,000 ± 40,000	22,000 ± 7,000	260,000 ± 10,000
Intradermal	1,100 ± 100	23,000 ± 8,000	4,400 ± 1,000	99,000 ± 30,000

[a] Groups of five mice received 500,000 BSA-pulsed DCs by various routes on day 1 followed by BSA protein boost on day 28 (Group 1) or DCs days 1 and 28 with BSA protein boost on day 56 (Group 2).

[b] Values are ng ^{125}I-BSA bound/ml of sera ± S.E.M. A positive result is defined as exceeding the mean +2 S.D. of a panel of normal control sera and is >60 ng/ml.

At a dose of 500,000 BSA-pulsed DCs, the antibody titers of three routes, intraperitoneal (i.p.), intravenous (i.v.) and intradermal (i.d.), were clearly positive and were comparable to each other four weeks after the initial cellular injection (Table 1). All three groups had titers which were much greater than the control mice who received MNCs exposed to BSA and washed three times (data not shown). Given a BSA challenge of 100 μg subcutaneously, the mice who received cells via the i.v. route had a log-fold greater antibody response to BSA than either the i.p. or i.d. routes seven days post-challenge. This is despite the fact that the

i.v. mice received no incomplete Freund's adjuvant (IFA) with the BSA challenge, while the i.p. and i.d. mice did receive IFA with the BSA. This is significant because naive mice show a greater antibody response at seven days to BSA in IFA than to straight BSA protein (700 ± 90 and 220 ± 60, respectively).

After receiving the initial cellular injection followed by a boost injection of cells four weeks later, the titers of all three groups were significantly higher than the groups who received only one injection of cells. The mice who received cells i.v. responded to the protein challenge the best, with antibody titers 6-fold greater than either the i.p. or i.d. mice. Seven days after being challenged with BSA, the antibody titers of the i.p. and i.d. groups approached that of the i.v. group. They were all the same order of magnitude, yet the i.v. group had a significantly higher titer, again without IFA in the challenge.

We tested different doses of DCs with the i.p. route (Table 2).

Table 2. Anti-BSA Antibody Response Elicited by Various Numbers
of BSA-Pulsed Dendritic Cells[a]

Cell Type and Dose	Group 1		Group 2	
	Day 28	Day 35	Day 56	Day 63
500,000 MNCs	2 ± 1[b]	$1,100 \pm 300$	210 ± 50	$2,400 \pm 1,000$
500,000 DCs	450 ± 200	$13,000 \pm 6,000$	$3,300 \pm 1,100$	$110,000 \pm 50,000$
250,000 DCs	510 ± 90	$1,100 \pm 900$	$27,000 \pm 10,000$	$61,000 \pm 8,000$
125,000 DCs	40 ± 20	$1,600 \pm 70$	110 ± 20	$17,000 \pm 5,000$

[a] Groups of five mice received BSA-pulsed cells by i.p. routes on day 1 followed by BSA protein boost on day 28 (Group 1) or DCs days 1 and 28 with BSA protein boost on day 56 (Group 2).

[b] Values are ng ^{125}I-BSA bound/ml of sera $\pm$ S.E.M. A positive result is defined as exceeding the mean +2 S.D. of a panel of normal control sera and is >60 ng/ml.

Given one injection of cells, the mice who received 250,000 or 500,000 DCs had positive antibody titers to BSA after 28 days, as seen in Table 2. The mice who received only 125,000 DCs and the control mice had negative titers after 28 days. When challenged with protein, every group of mice converted to a positive titer after seven days. The mice who received only 250,000 or 125,000 DCs had no advantage in antibody production over those who received MNCs. However, the mice who received 500,000 DCs produced antibodies at levels a log higher than the other mice. In unboosted mice, the dose of 125,000 DCs yielded lower antibody titers than the higher numbers of DCs, indicating a dose-dependent effect (day 28 values).

Twenty-eight days after the mice were boosted with the varying doses of DCs (Group 2), each group of mice had a positive titer to BSA. The group who received only 125,000 DCs had a titer actually lower than the control group who received only MNCs. The group who received 500,000 DCs had a log-fold greater titer than controls, and the group who received 250,000 DCs had a titer a log-fold greater than the 500,000 DC group. Seven days after protein challenge, all groups were definitely positive and significantly greater than the control group. At this timepoint, a dose-dependent effect is seen, with the best response seen with the group who received 500,000 cells.

Lymphoblastic transformation (LBT) assays were performed to document systemic T cell immunity to BSA following DC immunization. Specific lymphoblastic transformation to BSA was not elicited in any of these groups of mice; however, the splenic T cells did transform when re-exposed to fetal calf serum (FCS) at 1% and 10% as shown in Table 3.

Table 3. Lymphoblastic Transformation Response Elicited by BSA-Pulsed Dendritic Cells
Delivered by Various Routes

	Cell Type and Route										
Antigen (µg/ml)	MNCs i.p.			DCs i.p.			DCs i.v.			DCs i.d.	
Control[a]	130	±	40[b]	54 ±	30	26	±	5	33	±	5
BSA (100)	140	±	100	38 ±	20	40	±	10	33	±	6
BSA (30)	120	±	40	37 ±	20	34	±	6	38	±	9
BSA (10)	130	±	20	34 ±	20	50	±	10	59	±	10
FCS 10%	680	±	600	10,000 ±	5,000	3,300	±	1,000	690	±	300
FCS 1%	220	±	100	5,100 ±	3,000	580	±	400	110	±	30
FCS 0.1%						120	±	30	45	±	10
OVA[c] (100)	110	±	40	33 ±	7	20	±	3	24	±	3
Con A	74,000	±	20,000	2,000 ±	500	5,400	±	400	1,000	±	200

[a] Wells received 10^5 nylon wool enriched T cells and 5×10^5 syngeneic irradiated splenocytes as antigen-presenting cells.

[b] Values are cpm of ^{3}H-thymidine incorporated into cells ± S.E.M.

[c] OVA, ovalbumin.

The LBT assays of the three routes of administration of DCs in boosted groups at the maximum dose of 500,000 cells were compared four weeks after the last injection of cells. The group who received the cells i.p. had the greatest response to FCS, being two log-fold greater than the control group who received 500,000 MNCs. The group who received the cells i.v. had counts one log-fold greater than the controls, and the i.d. group had counts no greater than the control group. The counts of all groups decrease in parallel with the amount of FCS in the well. All groups responded well to con A and did not respond at all to ovalbumin.

The lymphoblastic transformation assay was also used to compare the efficacy of varying doses of dendritic cells to elicit systemic T cell immunity to BSA in boosted groups who

received cells i.p. Again, there was a good response to re-exposure to FCS without much response to BSA alone. A dose-dependent effect is seen in Table 4.

Table 4. Lymphoblastic Transformation Elicited by Various Numbers of BSA-Pulsed Dendritic Cells

Antigen (µg/ml)	Cell Type and Number[a]											
	500,000 MNCs			500,000 DCs			250,000 DCs			125,000 DCs		
Control[b]	130	±	40[c]	54	±	30	33	±	6	35	±	6
BSA (100)	140	±	100	38	±	20	30	±	6	32	±	10
BSA (30)	120	±	40	37	±	20	27	±	5	37	±	7
BSA (10)	130	±	20	34	±	20	50	±	10	47	±	10
FCS 10%	680	±	600	10,000	±	5,000	2,700	±	1,000	140	±	60
FCS 1%	220	±	100	5,100	±	3,000	500	±	200	55	±	20
FCS 0.1%							90	±	60	33	±	5
OVA[d] (100)	110	±	40	33	±	7	16	±	7	20	±	3
Con A	74,000	±	20,000	2,000	±	500	4,800	±	800	860	±	200

[a] All cells were injected intraperitoneally.

[b] Wells received 10^5 nylon wool enriched T cells and 5×10^5 syngeneic irradiated splenocytes as antigen-presenting cells.

[c] Values are cpm of ^{3}H-thymidine incorporated into cells ± S.E.M.

[d] OVA, ovalbumin.

An increasing response to FCS is obtained as the cell numbers increase. In the group that received only 125,000 DCs, counts are below that of background. The mice who received 250,000 DCs responded to FCS a log-fold greater than controls. All groups responded well to con A and were negative to ovalbumin.

These studies utilized dendritic cells exposed to a foreign protein (BSA) in overnight culture followed by injection of protein loaded cells for immunization. While many routes and doses have been examined in the literature, this is the first study to my knowledge where various routes were compared in one study. We have presented this data at the Keystone Symposium for Dendritic Cell Research (Taos, New Mexico, March 1995). Data which we have obtained regarding the optimal cellular dose and delivery route for protein-pulsed dendritic cell vaccines is applicable to dendritic cell immunization against a wide array of target antigens, including tumor-associated antigens.

Carcinoembryonic antigen (CEA) represents a reasonable tumor-associated antigen for a tumor vaccine against multiple human adenocarcinomas (breast, colon and non-small cell lung cancer) (14,15). The CEA gene has been cloned and inserted into a vaccinia virus genome (16,17). This recombinant vaccinia-CEA (rV-CEA) vaccine induces CEA-specific humoral and cellular immune responses in mice (17).

We have evaluated two strategies to develop a CEA-dendritic cell vaccine. First, we capitalized on the ability of the dendritic cells to take up, process and present antigen by

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exposing the cells to purified CEA protein in overnight culture. We have shown that CEA-pulsed dendritic cells can stimulate proliferation of CEA-immune T cells *in vitro*, which indicates intracellular processing and presentation of the antigen on the surface of the dendritic cells. The limited availability of purified tumor antigens including CEA requires protein loading overnight with concentrations of immunogen that are one to two logs lower than prior published studies or our BSA model. However, *in vitro* stimulation of CEA immune T cells by CEA pulsed dendritic cells suggested that *in vivo* studies were at least feasible. We were unable to induce reproducible antibody or cellular immunity to CEA in mice receiving CEA pulsed dendritic cells in various doses by i.v. and i.p. routes. Evidence for "priming" of the immune response can be seen in the tumor challenge studies below. Seven of eight mice challenged with 2×10^5 syngeneic CEA expressing tumor cells developed tumors (Figure 1), four of five mice given a single dose of CEA protein similarly developed tumors (Figure 2) while zero of eight animals developed tumors who received 500,000 CEA pulsed dendritic cells times two followed by a single dose of CEA protein (Figure 3). The requirement for large doses of immunogen and the weak immune response to CEA protein pulsed dendritic cells suggested we needed an alternate strategy to generate intracellular immunogen. Analysis of liposome systems documented their need for high concentrations of immunogen as well. We thus turned to CEA gene transduction of dendritic cells.

Figure 1. CEA-Pulsed Dendritic Cell Study

Naive Controls (n=8)
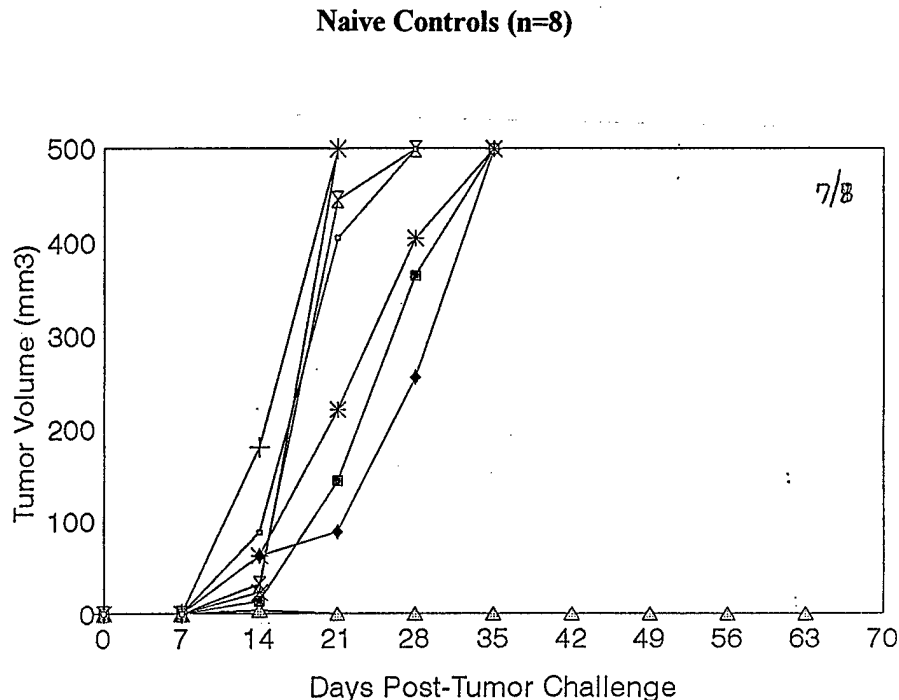
Control (unimmunized) mice received a subcutaneous injection of 2×10^5 MC38, CEA expressing tumor cells and had serial tumor measurements made over 63 days. Seven out of eight mice developed tumors.
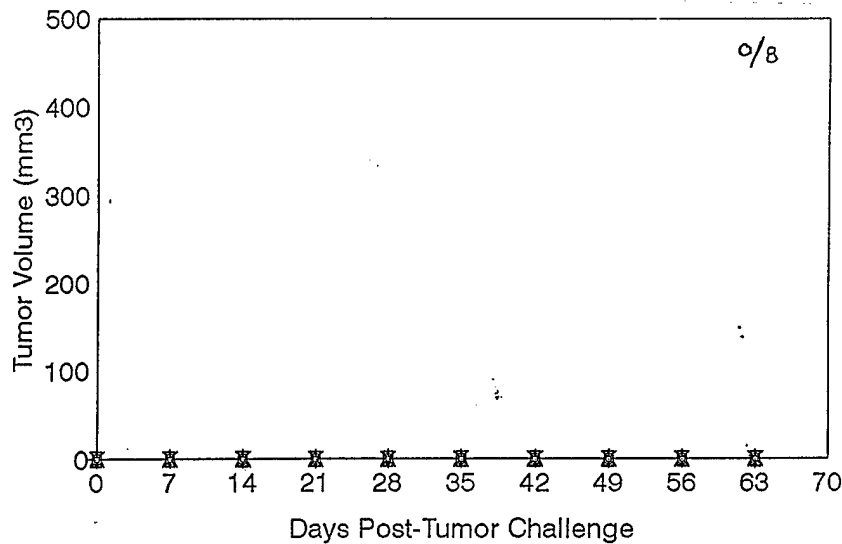
Figure 2. CEA-Pulsed Dendritic Cell Study

Protein Controls
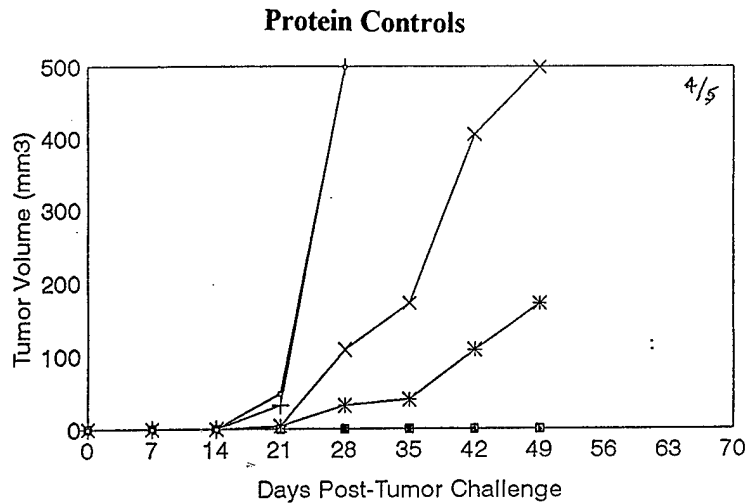
Five mice were immunized with a single dose of 100 µg of CEA protein subcutaneously and injected subcutaneously with 2 x 10⁵ MC38, CEA expressing tumor cells 28 days post-CEA injection and had serial tumor measurements made over 63 days. Four out of five mice developed tumors.

Figure 3. CEA-Pulsed Dendritic Cell Study

500,000 DCs IP x 2 injections; CEA protein SQ at 8 weeks

Eight mice received intraperitoneal injections of 500,000 CEA pulsed dendritic cells on days 1 and 29. They then received a CEA protein boost (100 µg) subcutaneously on day 57 and were challenged with 2 x 10⁵ MC38, CEA expressing tumor cells subcutaneously on day 85. Serial tumor measurements were made over 63 days. Zero out of eight mice developed tumors.

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We evaluated the relative transducibility of human hematopoietic cells (T cells, B cells, monocytes, and dendritic cells) by a panel of vectors encoding a luciferase reporter gene (recombinant adenovirus, DNA/liposome complexes, RNA/liposome complexes, and DNA/adenovirus conjugates). Recombinant adenovirus was the only vector demonstrating the ability to transduce unstimulated human hematopoietic cells; and dendritic cells were the most transducible subpopulation with luciferase activity 8 to 40-fold that of T cells, B cells or monocytes (Table 5). Similar results were observed with recombinant adenovirus using murine dendritic cells. Each dendritic cell purification was verified by stimulatory activity in the mixed lymphocyte reaction (30 to 100-fold more potent than MNCs). A recombinant adenovirus encoding human CEA (Ad-CEA) was constructed and verified by transduction of carcinoma cells with CEA expression detected by radiolabeled CEA-specific monoclonal antibody binding. Murine dendritic cells transduced with Ad-CEA were shown to stimulate lymphoblastic transformation of CEA-immune T cells *in vitro*.

Table 5. Dendritic Cell Transduction

|	Luciferase reporter gene			
	Recombinant Adenovirus	**DNA/ Liposome**	**RNA/ Liposome**	**Adenovirus Conjugate**
Human breast cancer	1,000,000	51,000	11,000	50,000
Human T cells	190	32	30	30
Human B cells	180	32	30	31
Human monocytes	990	29	30	29
Human dendritic cells	7,900	31	31	30
Mouse spleen	2,900	41	29	30

We have begun analysis of CEA gene transduction vaccine efforts by examining the effects of i.v. injection of the Ad-CEA for *in vivo* transduction of antigen presenting cells. As seen in Figure 4, mice given a single dose of i.v. Ad-CEA produced very high antibody responses (4,000-7,000 ng/ml) with anamnestic response to Ad-CEA (10,000-20,000 ng/ml) and even higher if given a booster of CEA protein (35,000-50,000 ng/ml). The same profound antibody response occurred if the vector (Ad-CEA) was given i.p. (Figure 5). However, immunoprotection against tumor challenge with syngeneic CEA expressing tumor cells did not occur (Figure 6).

Figure 4. Anti-CEA Ab Response Among Mice Immunized with AdCEA Via I.V. Route
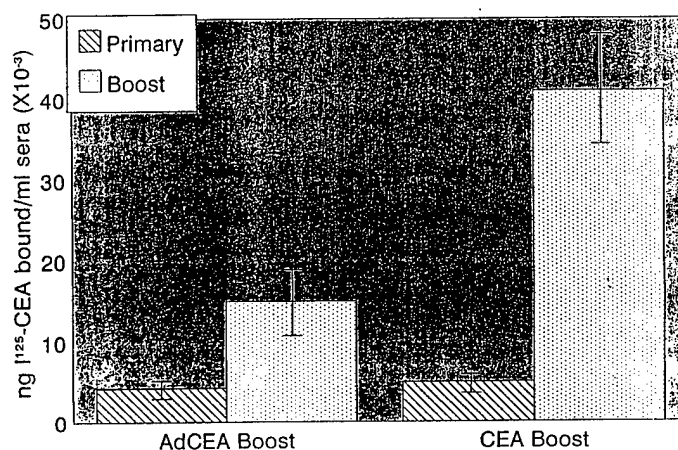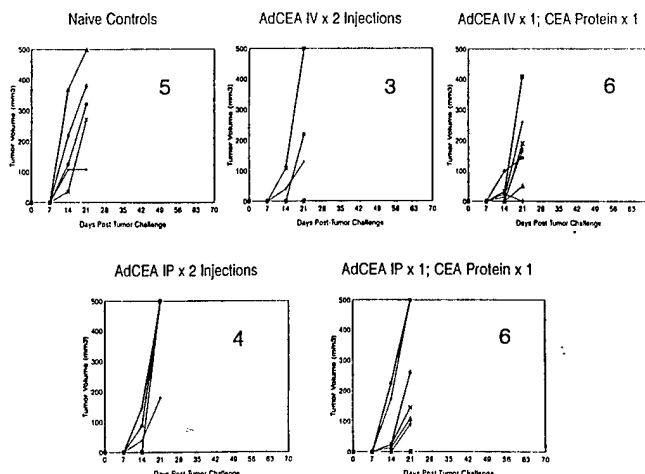

Two groups of seven C57BL/6 mice received 10^9 pfu of replication incompetent adenovirus encoding CEA (AdCEA) by i.v. injection on day 1. On day 29, one group was boosted with 10^9 pfu of AdCEA i.v. whereas the other group was boosted with 25 µg of CEA protein by i.m. injection. Mice were bled on days 29 and 43 to assess primary and boosted anti-CEA antibody responses, respectively. Values are the mean ± S.E.M. for seven mice.

Figure 5. Anti-CEA Ab Response Among Mice Immunized with AdCEA Via I.P. Route
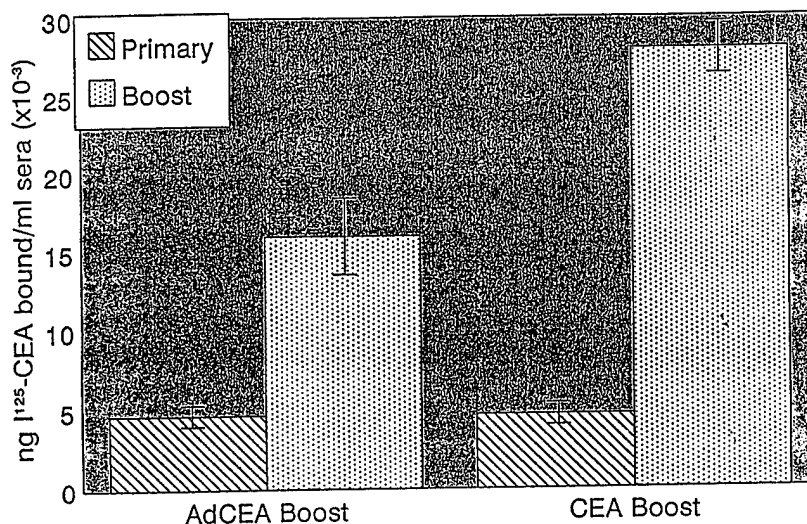
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Figure 6. Tumor Challenge Outcome Among Mice Immunized With Recombinant
Adenovirus Encoding CEA With or Without CEA Protein Boosting

Groups of seven C57BL/6 mice were immunized as follows: 1) 10^9 pfu of AdCEA i.v. on days 1 and 29 (panel B); 2) 10^9 pfu of AdCEA i.v. on day 1 and 25 μg of CEA protein i.m. on day 29 (panel C); 3) 10^9 pfu of AdCEA i.p. days 1 and 29 (panel D); or 4) 10^9 pfu of AdCEA i.p. day 1 and 25 μg of CEA protein i.m. day 29 (panel E). These mice as well as five naive controls (panel A) were challenged with 2×10^5 MC38-CEA cells on day 43. Serial tumor measurements and the total number of tumor bearing mice in each group are shown.

Immunization with dendritic cells transfected ex vivo with recombinant adenovirus encoding human CEA

Dendritic cells from mouse spleen were enriched as described above. During the overnight adherence step of the isolation procedure, these cells were incubated with recombinant adenovirus encoding human CEA (AdCEA) at a M.O.I. of 5i1. Non-adherent cells enriched for dendritic cells were harvested, washed extensively to remove free AdCEA, and injected into syngeneic mice. An equal volume of supernatant from the last wash was injected into control mice to control for immunization due to trace quantities of free AdCEA coinjected with the AdCEA transfected dendritic cells. In a representative experiment, groups of five C57BL/6 mice received 10^6 enriched dendritic cells transfected by AdCEA by either i.v. or i.p. injection day 1 and day 29 with sera obtained day 29 and 57 for anti-CEA antibody response.
As shown in Table 6, anti-CEA antibody responses were clearly demonstrated after primary immunization in 3 of 5 mice receiving AdCEA dendritic cells by i.v. injection. Following booster immunization 3 of 5 remained positive with only a modest increase in the magnitude of the responses. The supernatant control mice and mice receiving AdCEA dendritic cells i.p. had no detectable anti-CEA antibody response. The magnitude of the anti-CEA antibody response among mice immunized with AdCEA dendritic cells was less than that observed following immunization with an optimal dose of free AdCEA. Efforts to demonstrate CEA-specific T cell proliferative responses and/or protection against challenge with syngeneic CEA-expressing tumor cells among mice receiving AdCEA transfected dendritic cells were unsuccessful despite repeated experiments (data not shown).

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Table 6. Anti-CEA Antibody Response among Mice Receiving AdCEA Transfected Dendritic Cells By I.V. Or I.P. Injection

A. Sera obtained day 29

	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
AdCEA DC's I.V.	1400	550	2800	0	0
Supernatant Control I.V.	0	0	0	0	0
AdCEA DC's I.P.	0	0	0	0	0
Supernatant Control I.P.	0	0	0	0	0

B.Sera Obtained Day 57.

	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
AdCEA DC's I.V.	1300	4300	2900	0	0
Supernatant Control I.V.	0	0	0	0	0
AdCEA DC's I.P.	0	0	0	0	0
Supernatant Control I.P.	0	0	0	0	0

During the last year of this grant, our success in the field of polynucleotide immunization against tumor associated antigens[18] led us to examine strategies to augment the immune response elicited by DNA-mediated immunization through enhanced antigen delivery to dendritic cells in vivo[19]. In this regard, there has been increasing interest in the *in vivo* delivery of plasmid DNA encoding a relevant antigen as a novel approach to vaccination, hereafter referred to as "polynucleotide immunization".[20,21,22] The technique of direct intramuscular injection of plasmid DNA encoding specific antigens has elicited humoral and cellular immune responses to a variety of infectious agents including influenza, hepatitis B, human immunodeficiency virus, and others.[20,21,22] An alternate approach to polynucleotide immunization described by Tang *et al.* involves bombardment of the skin with gold microprojectiles coated with plasmid DNA encoding a specific antigen. This particle bombardment strategy has elicited humoral immune responses to human growth hormone[23,24] and influenza hemagglutinin as well as protection against influenza virus challenge.[25]

To examine the ability of polynucleotide immunization to achieve specific antitumor immunity, we have constructed a plasmid DNA encoding the full-length complementary DNA (cDNA)

for human carcinoembryonic antigen (CEA) under transcriptional regulatory control of the cytomegalovirus early promoter/enhancer.[26] This plasmid delivered by intramuscular (i.m.) injection can function as a polynucleotide vaccine to elicit CEA-specific humoral and cellular immune responses as well as protection against syngeneic, CEA-expressing colon carcinoma cells.[26,27,28] These effects were comparable to the immune response and immunoprotection achieved with a recombinant vaccinia virus encoding CEA.[26]

When considering possible strategies to achieve immune amplification, we utilized recent observations pertinent to antigen presenting cell (APC) mechanisms. The success of cutaneous polynucleotide immunization by particle bombardment is thought to derive largely from the presence of dendritic Langerhans cells, highly potent antigen presenting cells, within the skin. Resting Langerhans cells are weak accessory cells for the sensitization phase of primary T cell dependent immune responses.[29,30] However, Langerhans cells can mature immunologically in bulk epidermal culture and acquire most of the features of lymphoid dendritic cells including increased levels of major histocompatibility complex (MHC) class I and class II molecule expression. In this regard, granulocyte macrophage colony stimulating factor has been identified as the principal mediator of immunologic maturation of Langerhans cells in culture.[29,30] Furthermore, GM-CSF plays an important role in determining the distribution and differentiated state of Langerhans cells *in vivo*.[31] Thus, we reasoned that cutaneous delivery of GM-CSF cDNA by particle bombardment with resultant local GM-CSF expression would induce immunologic maturation of Langerhans cells enhancing their ability to present co-delivered antigens. Therefore, we have evaluated co-delivery of cDNA encoding GM-CSF with cDNA encoding CEA as a potential augmentation strategy for cutaneous polynucleotide immunization.

The ability of GM-CSF co-expression to enhance the humoral and cellular immune response elicited by CEA-polynucleotide immunization via particle bombardment was evaluated using 2 µg doses of plasmid DNA encoding CEA (pGT37). Groups of five mice received 2 µg of plasmid DNA encoding GM-CSF (pGM-CSF) either mixed with each dose of pGT37 (CEA) or delivered by particle bombardment three days prior to each dose of pGT37 (CEA) at the same site. Control groups received pGT37 (CEA) alone or 2 µg of pCAT three days prior to each dose of pGT37 (CEA). Mice were immunized on days 1 and 29 with sera and spleens obtained two weeks after the last immunization for anti-CEA antibody and lymphoblastic transformation assays. Table 7 provides the anti-CEA antibody response with all five mice receiving pGT37 (CEA) alone demonstrating an anti-CEA antibody response whereas delivery of pGM-CSF three days before each dose of pGT37 (CEA) elicited anti-CEA antibody in all five mice with a mean level twice that of mice receiving pGT37 (CEA) alone. A one-way analysis of variance applied to ranks revealed that pretreatment with pGM-CSF significantly enhanced the antibody response compared to pGT37 (CEA) alone or pretreatment with plasmid DNA encoding chloramphenicol acetyltransferase (pCAT) ($p=0.04$ and $p<0.01$, respectively). Concurrent administration of pGM-CSF diminished the immune response with only one of five mice positive for anti-CEA antibody ($p<0.01$). Delivery of an irrelevant plasmid (pCAT) three days before each dose of pGT37 (CEA) to control for non-specific injury related to particle bombardment produced no enhancement of antibody response to CEA.

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Table 7   **Effect of pGM-CSF co-expression on the anti-CEA antibody response to polynucleotide immunization by particle bombardment[a]**

pGM-CSF Administration	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
None	59[b]	70	93	60	109
pGM-CSF days 1 and 29	36	0	0	0	0
pGM-CSF days -2 and 26	183	216	65	159	149
pCAT days -2 and 26	0	50	106	115	0

[a] Groups of five mice received 2 μg of pGM-CSF either mixed with each dose of pGT37 (CEA) or delivered by particle bombardment three days prior to each dose of pGT37 (CEA). Control mice received pGT37 (CEA) alone or 2 μg of pCAT three days prior to each dose of pGT37 (CEA). All mice received 2 μg doses of pGT37 (CEA) on days 1 and 29 with sera obtained two weeks after the last immunization.

[b] Results expressed as ng of ^{125}I-CEA bound/ml of. A positive result is defined as exceeding 2 S.D. above the mean of normal mouse sera and is >15 ng/ml.

The same experimental groups of mice were analyzed with regard to splenic lymphoblastic transformation. CEA-specific lymphoblastic transformation data from the five mice receiving pGM-CSF three days prior to each dose of pGT37 (CEA) is provided in Table 8. All five mice demonstrated dose-dependent lymphocyte proliferative responses to human CEA with peak stimulation ratios ranging from 34 to 692. All mice failed to respond to ovalbumin included as a control antigen with stimulation ratios ranging from 0.7 to 1.2, and mitogen responses were intact. Complete data from these five mice have been provided as mean cpm with the standard error to illustrate the reliability of these assays as well as the dose-dependent nature of the CEA-specific immune responses in the context of appropriate positive and negative controls.

We have never seen positive cellular immune responses to CEA in unimmunized animals or animals immunized with control plasmids.[26,27,28] All particle bombardment groups of mice were assayed with the same panel of antigens and mitogens over the same range of concentrations. To facilitate comparison of data between groups of mice, the results have been provided as stimulation ratios for cells stimulated with 10 μg/ml of CEA (Table 9). Three of five mice receiving the CEA plasmid (pGT37) alone demonstrated moderate CEA-specific lymphoblastic transformation whereas administration of pGM-CSF three days prior to pGT37 (CEA) elicited marked CEA-specific lymphocyte proliferative responses in five of five mice. A one-way analysis of variance applied to ranks demonstrated that this difference was significant (p=0.001). Concurrent delivery of pGM-CSF diminished the immune response producing no evidence of lymphoblastic transformation (p=0.02). Pretreatment with pCAT produced no enhancement of lymphocyte response to CEA. In all instances, response to

Table 8. CEA-specific lymphoblastic transformation elicited by particle bombardment with pGM-CSF three days prior to pGT37 (CEA)[a]

	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
Control	18 ± 3b	20 ± 5	14 ± 3	15 ± 3	13 ± 2
CEA 10 μg/ml	620 ± 100	3000 ± 500	4700 ± 900	4400 ± 1000	9000 ± 3000
CEA 3 μg/ml	290 ± 50	2600 ± 500	3900 ± 1000	790 ± 300	6700 ± 600
CEA 1 μg/ml	160 ± 50	140 ± 50	88 ± 10	120 ± 40	2100 ± 800
OVA 100 μg/ml	14 ± 2	14 ± 2	14 ± 3	13 ± 3	16 ± 2
CON A	14000 ± 200	12000 ± 200	8800 ± 40	7900 ± 200	33000 ± 900

[a] Mice (5) received 2 μg of pGM-CSF by particle bombardment three days prior to each 2 μg dose of pGT37 (CEA) on days 1 and 29 with splenic T cells obtained two weeks after the last immunization.

[b] Results are the mean cpm's of quadruplicate wells ± SEM.

Table 9. Effect of pGM-CSF on the CEA-specific lymphoblastic transformation response to polynucleotide immunization by particle bombardment[a]

pGM-CSF Administration	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
None	9[b]	17	1	1	12
pGM-CSF days 1 and 29	1	1	1	1	1
pGM-CSF days -2 and 26	34	150	336	294	689
pCAT days -2 and 26	2	1	1	1	2

[a] Groups of five mice were immunized with 2 μg doses of pGT37 (CEA) on days 1 and 29 with or without co-delivery of pGM-CSF or pCAT. Splenic T cells were obtained two weeks after the last immunization.

[b] Results are the mean stimulation ratio of quadruplicate wells stimulated with 10 μg/ml of baculovirus recombinant human CEA. A positive response is defined as a stimulation ratio ≥2.

control ovalbumin was negative and mitogen responses were brisk. Thus, pGM-CSF delivered by particle bombardment three days before each dose of pGT37 (CEA) produces substantial enhancement of the cellular immune response and modest enhancement of the humoral

immune response to CEA. This effect is dependent upon the GM-CSF cDNA since pretreatment with pCAT did not augment the immune response to CEA.

Discussion

Cutaneous particle bombardment utilizing gold microbeads coated with plasmid DNA is an alternate delivery mechanism for polynucleotide immunization. The efficacy of this approach is thought to derive in part from delivery of antigen to epidermal Langerhans cells, potent antigen presenting cells within the dendritic cell family.[24] Prior studies have demonstrated humoral immune responses to viral antigens as well as protection against viral challenge in a variety of species.[23,24,25] This strategy has not previously been analyzed regarding generation of an immune response to a tumor-associated antigen.

We evaluated the capacity of GM-CSF cDNA co-delivery to augment the CEA-specific immune response elicited by cutaneous particle bombardment with plasmid DNA encoding CEA (pGT37). The GM-CSF cytokine was selected for use in this system based upon evidence that GM-CSF is the principle mediator of immunologic maturation of epidermal Langerhans cells *in vitro*[29,30] and plays an important role in determining the distribution and differentiated state of Langerhans cells *in vivo*.[31] Furthermore, Dranoff *et al.* demonstrated that transduction of tumor cell vaccines with GM-CSF cDNA could enhance the induction of potent antitumor immunity to poorly immunogenic tumors.[32] We examined delivery of pGM-CSF three days before each dose of plasmid DNA encoding CEA versus concurrent delivery of both plasmids based upon evidence that protein expression by gene gun delivery and immune effects on Langerhans cells would require some time to occur. Augmentation of CEA-specific lymphoblastic transformation and antibody response was observed when pGM-CSF administration antedated CEA plasmid delivery while concurrent delivery of both plasmids prevented the CEA-specific immune response from occurring. The inhibition of immune response might be related to competition between the two genes for expression. Also, gene expression following particle bombardment is transient with most of the expression being lost within three days due to normal sloughing of epidermal keratinocytes.[24,33] Thus, simultaneous delivery of pGM-CSF and the CEA plasmid would not be expected to produce immunologic maturation of the Langerhans cells until the majority of CEA expression by keratinocytes was lost. Enhancement of the immune response to plasmid DNA encoding CEA by pretreatment with pGM-CSF appears dependent upon the GM-CSF cDNA rather than nonspecific effects related to the trauma of particle bombardment as pretreatment with pCAT produced no augmentation.

Thus, pretreatment with plasmid DNA encoding GM-CSF augments the immune response to cutaneous polynucleotide immunization by particle bombardment, perhaps through local immunologic maturation of Langerhans cells. The strategy of augmenting the immune response to cutaneous polynucleotide immunization by co-delivery of accessory molecule cDNAs is applicable to a broad range of vaccine applications including infectious agents and other tumor-associated antigens. Studies are ongoing to evaluate other cytokines, growth

factors and co-stimulatory molecules as well as the molecular configuration and timecourse for their delivery.

Plasmid DNAs for vaccination

Plasmid constructs: The gene for full-length human CEA[34] was used to construct an expression plasmid from the vector pCDNA3 (Invitrogen, San Diego, CA, USA), in which transcription is driven by the cytomegalovirus early promoter/enhancer.[26,27,28] For this construction, the neomycin resistance gene, the ampicillin resistance gene, and nonessential viral sequences were deleted from pCDNA3. The Tn903 kanamycin resistance gene from pUC4K (Pharmacia, Piscataway, NJ, USA) was inserted to allow selective propagation in *E. coli*. This expression vector is called pGT36.[28] The human CEA cDNA was then cloned into this modified eukaryotic expression vector to produce pGT37 (CEA) as previously described.[28] As control plasmids, we utilized pCDNA-CAT (Invitrogen) which contains the chloramphenicol acetyltransferase reporter gene in the pCDNA3 vector (designated pCAT) and pUC18 (Gibco, BRL, Grand Island, NY, USA), a procaryotic cloning vector containing no eukaryotic expression cassettes.

Murine GM-CSF cDNA was provided by Dr. Nicholas Gough (WEHI, Melbourne, Australia).[35] To enhance gene expression, a fragment corresponding to base pairs 131-620 of the murine GM-CSF cDNA was amplified via polymerase chain reaction. This fragment, representing the coding region without the 5′ and 3′ untranslated regions, was verified by DNA sequencing and subcloned into a pNAss vector (Clontech, Palo Alto, CA, USA) with the human cytomegalovirus promoter to give pGM-CSF (Figure 7).
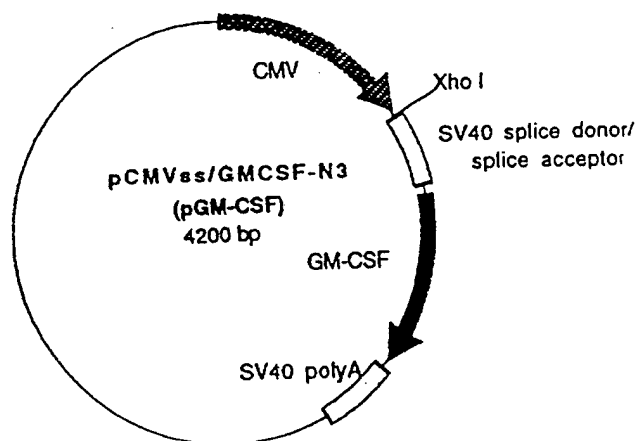

Figure 7.
Plasmid purification: *E. coli* strain DH5α (Gibco BRL, Grand Island, NY, USA) carrying the pGT37, or pGM-CSF plasmids was grown in Terrific Broth (Gibco BRL). Antibiotic selection employed 50 μg/ml of kanamycin for pGT37 (CEA), pGT59 (B7-1) and pGT64 (CEA/B7-1) whereas 100 μg/ml of ampicillin was used for pGM-CSF. Plasmids were purified

using Qiagen Plasmid Mega Kits (Qiagen, Inc., Chatsworth, CA, USA) according to the manufacturer's instructions, with one Qiagen tip 2500 used for each liter of *E. coli* culture. Endotoxin was removed by extraction with Triton X-114 (Sigma, St. Louis, MO, USA) as previously described.[28] The DNA was precipitated in large lots (5 mg) and stored at -70°C as pellets. For experimental use, the DNA was resuspended in sterile saline at a concentration of 5 mg/ml and stored in aliquots at -20°C for use in immunization protocols.

Functional validation of plasmid DNA: Verification of pGM-CSF expression was performed by transfection of murine B16 melanoma cells via gene gun particle bombardment. For this analysis, murine GM-CSF expression was quantitated by ELISA assay (Pharmingen, San Diego, CA, USA) of cell culture supernatant obtained 18 hours after transfection.

Reagents: Native human CEA purified from hepatic metastases of human colonic adenocarcinoma was obtained from Vitro Diagnostics, Littleton, CO, USA.[36] Baculovirus recombinant human CEA was kindly provided by MicroGeneSys, Meriden, CT, USA. Ovalbumin, concanavalin A (con A), and spermidine were obtained from Sigma Chemical Company. Polystyrene beads (6.4 mm) were obtained from Precision Plastic Ball (Chicago, IL, USA). Gold beads (0.9 μm) were obtained from Degussa, South Plainfield, NJ, USA. Tefzel tubing was obtained from McMaster-Carr, Chicago, IL, USA.

Cutaneous inoculation by particle bombardment: The preparation and immunization techniques for cutaneous immunization have been described previously.[24,25] In brief, eighty micrograms of either pGT37 (CEA), pCAT, pGM-CSF, or pGT37 (CEA) and pGM-CSF were added to a microcentrifuge tube containing forty milligrams of 0.9 micron gold beads suspended in 100 mM spermidine. While gently vortexing the tube, 400 microliters of 2.5M CaCl$_2$ were added to precipitate the DNA onto the beads and the tube was allowed to stand for ten minutes to complete the precipitation. The DNA coated beads (2 μg of DNA per mg of gold) were pelleted by a ten second spin and the supernatants were removed. The gold/DNA pellets were washed twice by vortexing in 1 ml ethanol, microcentrifuging ten seconds, and removing supernatants. The gold/DNA beads were transferred to a 15 ml culture tube, resuspended in 5.7 ml of ethanol to give 7 mg of gold/DNA per ml of ethanol. Sonication for ten seconds in a bath sonicator generated a uniform gold suspension. Using a syringe attached by an adapter, this suspension was drawn into a 30 inch length of Tefzel tubing, one milliliter (7 mg of gold/DNA) of suspension filling 7 inches of tubing, yielding 1 mg gold/DNA per inch of tubing. The tubing was then transferred into a tube turner (Agracetus, Inc.). After allowing the gold beads to settle, the ethanol was slowly drawn off and the turner was rotated for 30 seconds, smearing the gold/DNA around the inside of the tubing. The residual ethanol was removed by passing nitrogen through the tubing for three minutes. The tubing was cut into 1/2 inch long tubes (equal to one immunization dose) and the tubes were loaded into the Accell gene delivery device. Six- to eight-week old female C57BL/6 mice (Harlan/Sprague/Dawley, Indianapolis, IN, USA) were anesthetized as described above, and their abdomens were clipped. At adjacent sites on each abdomen, two doses of gold/DNA particles were delivered by a helium blast at a pressure of 400 pounds per

square inch. Each site received one microgram of DNA (one microgram for each vector in co-delivery) on half a milligram of gold.

Lymphoblastic transformation: This assay was performed as previously described.[26,27,28] Stimulated cells received baculovirus recombinant human CEA over a range of concentrations (1-10 ug/ml); ovalbumin (100 ug/ml) as a negative control antigen; or concanavalin A (con A) at 5 μg/ml as a positive control mitogen. The range of CEA concentrations described above provided optimal stimulation in our prior studies of pGT37 (CEA) immunized mice.[28] The stimulation ratio was calculated as mean cpm of the stimulated cells divided by mean cpm of the control cells. A positive response was defined as a stimulation ratio ≥ 2.0.

Antibody assay: Antihuman CEA antibody was quantitated using a double antigen immunoradiometric assay as previously described.[26,37] Briefly, polystyrene beads were coated with purified native human CEA (2 μg/bead) in phosphate buffered saline (PBS), washed three times with PBS containing 1% bovine serum albumin and stored in wash buffer at 4°C until use. Twenty microliters of mouse sera (normal control postvaccination) were diluted to 100 ul with PBS containing 1% bovine serum albumin and incubated with a single coated bead (in duplicate) for 2 hours on a laboratory oscillator at room temperature, washed with PBS and incubated with 100 μl of ^{125}I-labeled native human CEA (approximately 10^6 cpm per μg) at 2 μg/ml for 1 hour, rewashed with PBS and counted on a Micromedic automatic gamma counter. Background nonspecific binding of approximately 1% of the available ^{125}I-CEA was subtracted from cpm bound and the nanograms of CEA bound to the bead per ml of sera was calculated from the known specific activity of the ^{125}I-CEA. A positive response (>15 ng/ml) has been defined as exceeding 2 S.D. above the mean value of ten normal mouse sera.

Co-delivery of GM-CSF by intramuscular polynucleotide immunization in an effort to augment immune response through enhanced antigen presentation by dendritic cells

The mechanisms responsible for induction of immune response by intramuscular polynucleotide immunization have not been clearly delineated.[18] One hypothesis calls for plasmid DNA injection to elicit a nonspecific inflammatory response within the muscle that serves to recruit "professional" antigen-presenting cells to the injection site. These "professional" antigen-presenting cells then process and present antigen synthesized and released by injured myocytes. In support of this hypothesis, we have observed an inflammotry track in muscle within 72 hours of plasmid DNA injection. Furthermore, other investigators[38] have shown that mammalian immune systems can recognize bacterial DNA because it contains unmethylated CpG dinucleotides resulting in potential adjuvant effects. Bacterial DNA is a known contaminant of plasmid DNA preparations grown in *Escherischia coli and* may be responsible for this early local inflammatory reaction. We have also demonstrated that combining 50 μg of irrelevant plasmid DNA-encoding chloramphenicol acetyltransferase with a low dose (1 μg) of pCEA for i.m. injection significantly augments the CEA-specific immune response, as illustrated in Figure 8. This observation is consistent with augmentation of CEA antigen presentation secondary to an enhanced local inflammatory response induced by irrelevant plasmid DNA.
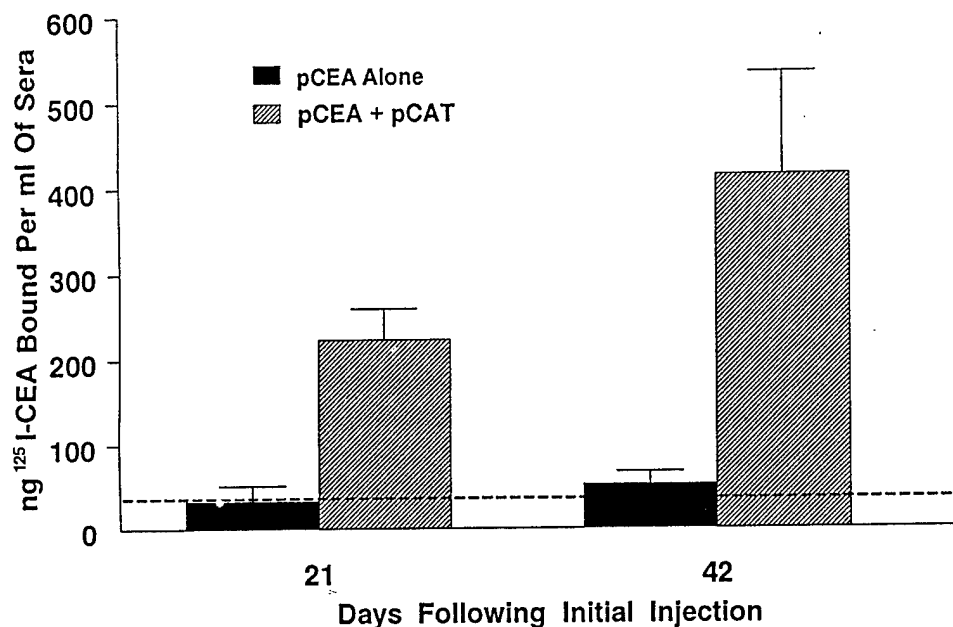
22

Figure 8. Augmentation of the anti-CEA antibody response to CEA polynucleotide immunization by co-delivery of irrelevant plasmid DNA. Groups of seven mice received low doses (1 μg) of plasmid DNA encoding CEA (pCEA) by i.m. injection alone or mixed with 50 μg doses of irrelevant plasmid DNA encoding chloramphenicol acetyltransferase (pCAT) for two injections three weeks apart. Sera for anti-CEA antibody response were obtained 21 and 42 days following the initial injection. Results are reported as ng of ^{125}I-CEA bound per ml of sera with the dashed line at 35 indicating the threshold for a positive result.

If induction of the immune response following intramuscular polynucleotide immunization indeed depends on antigen presentation by "professional;" antigen presenting cells recruited to the injection site, then co-delivery of cDNA encoding GM-CSF might augment the immune response through local recruitment and activation of dendritic cells. Based partly upon our success with co-delivery of GM-CSF cDNA in polynucleotide immunization administered by cutaneous particle bombardment described above, we evaluated this strategy in the context of intramuscular polynucleotde immunization. For these studies, we selected doses of pCEA (plasmid encoding CEA) and administration schedules which we had previously shown to be below the threshold for elicitation of CEA-specific antibody response and anti-tumor effects to allow any augmentation by GM-CSF co-delivery to be demonstratable.

In the first study groups of seven C57BL/6 mice received 1 μg of pCEA alone; 1 μg of pCEA mixed with 50 μg of GM-CSF; 1 μg of pCEA mixed with 50 μg of pCAT (plasmid encoding chloramphenicol acetyl transferase as a negative control); or an equimolar dose (1.2 μg) of a dual expression plasmid encoding CEA and GM-CSF administered by IM injection days 1 and 15. On day 29 sera was obtained for anti-CEA antibody response and all animals were tumor challenged with 2.5×10^5 syngeneic colonic adenocarcinoma cells expressing human CEA (MC38-CEA cells). As shown in Table 10, there was no significant differences between the anti-CEA antibody responses among the four groups.

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Table 10. Anti-CEA antibody response among mice receiving pCEA with or without GM-CSF cDNA co-delivery by intramuscular injection

	pCEA 1μg	pCEA + pGM-CSF 50μg	pCEA + pCAT 50μg	pCEA/GM-CSF 1.2μg
Mouse 1	23	17	37	8
Mouse 2	4	220	58	27
Mouse 3	24	45	16	27
Mouse 4	19	37	8	19
Mouse 5	24	14	9	7
Mouse 6	432	17	31	62
Mouse 7	22	25	19	14
Mean ± SEM	78 ± 60	17	25 ± 7	23 ± 7

Results are ng of ^{125}I-CEA bound per ml of sera with values > 26 representing a positive result.

As illustrated in Figure 9, there was perhaps a trend toward improved protection against tumor challenge among mice receiving the dual expression plasmid encoding CEA and GM-CSF.
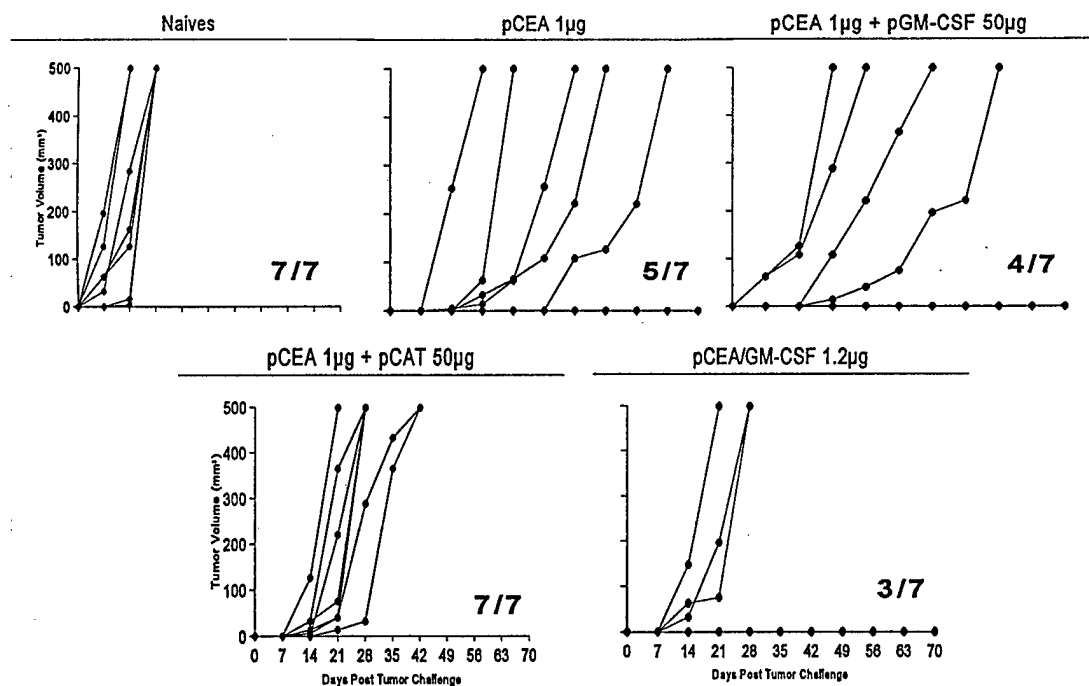

Figure 9. Tumor challenge outcome among mice receiving pCEA with or without GM-CSF cDNA co-delivery by intramuscular injection.

In the second study, groups of seven C57BL25/6 mice received 10 μg of pCEA alone; 10 μg of pCEA mixed with 50 μg of GM-CSF; 10 μg of pCEA mixed with 50 μg of pCAT; or an equimolar dose (12 μg) of a dual expression plasmid encoding CEA and GM-CSF administered by IM injection day 1. On day 22, sera were obtained for anti-CEA antibody response and all animals were tumor challenged with 2.5×10^5 MC38-CEA cells subcutaneously. As shown in Table 11, the anti-CEA antibody response was greater among mice receiving the dual expression plasmid (pCEA/GM-CSF) as compared to pCEA alone or pCEA mixed with pGM-CSF. However, co-delivery of irrelevant plasmid DNA (pCAT) produced a comparable enhancement of the anti-CEA antibody response. As illustrated in Figure 10, there was no significant difference between the degree of protection against tumor challenge among the five groups of mice in this study. Thus, although co-delivery of GM-CSF cDNA significantly enhances the humoral and cellular response to polynucleotide immunization by cutaneous particle bombardment, we were unable to demonstrate clear enhancement of anti-CEA antibody response or tumor protection with co-delivery of GM-CSF cDNA by i.m. injection.
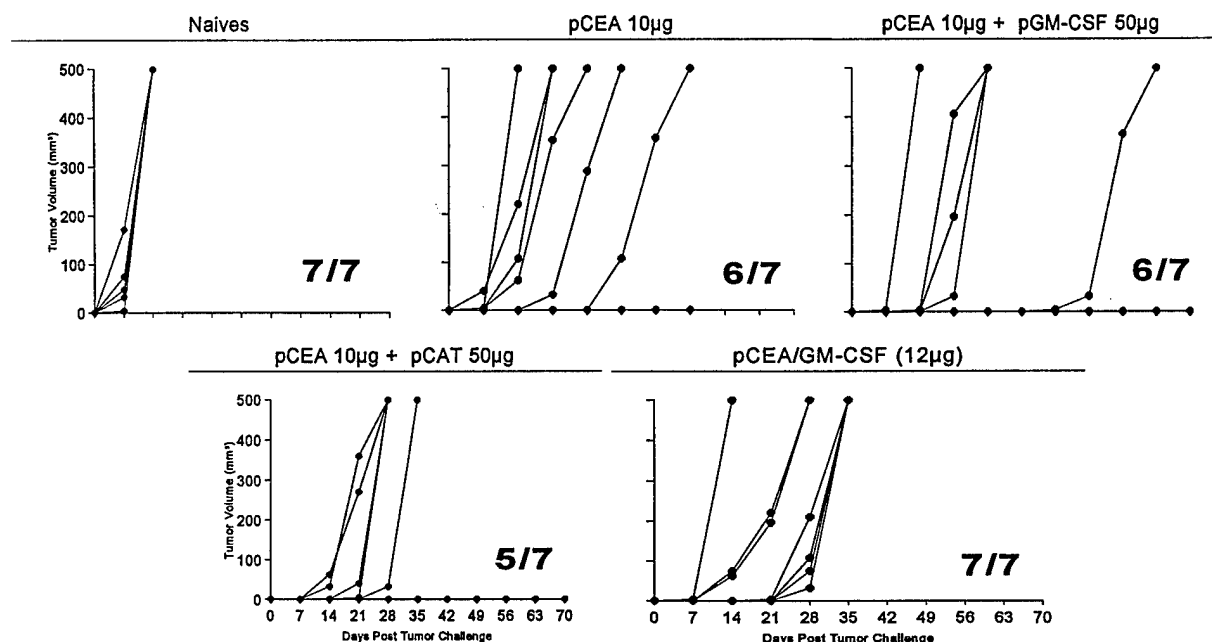

Figure 10.   Tumor challenge outcome among mice receiving pCEA with or without GM-CSF cDNA co-delivery by intramuscular injection.

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Table 11. Anti-CEA antibody response among mice receiving pCEA with or without GM-CSF cDNA co-delivery by intramuscular injection

	pCEA 1µg	pCEA + pGM-CSF 50µg	pCEA + pCAT 50µg	pCEA/GM-CSF 1.2µg
Mouse 1	19	0	329	33
Mouse 2	16	11	167	31
Mouse 3	0	18	191	467
Mouse 4	232	8	87	421
Mouse 5	52	58	302	436
Mouse 6	36	2	107	132
Mouse 7	63	21	34	28
Mean ± SEM	60 ± 30	17 ± 7	174 ± 41	221 ± 79

Results are ng of ^{125}I-CEA bound per ml of sera with values > 26 representing a positive result.

Conclusions:

These studies have demonstrated the feasibility of studying freshly isolated human blood or murine splenic dendritic cells. Overnight protein loading generated dendritic cell vaccines which were quite effective at induction of humoral and cellular immunity in a model system (BSA). However, the strategy produced weak immune responses using a tumor antigen (CEA) presumably due to requirements for high concentrations of the loading immunogen. We have developed a means to induce CEA expression in dendritic cells using a gene transfer vector (replication defective adenovirus [AdCEA]). The AdCEA alone produced intense antibody response to CEA following i.v. and i.p. injection but no tumor protection. Studies of AdCEA transfected dendritic cells demonstrated anti-CEA antibody responses following i.v. injection but not following i.p. delivery. Although these anti-CEA antibody responses were readily detectable after primary immunization, they boosted only modestly and were of lower magnitude than those observed following immunization with an optimal dose of free AdCEA alone. Furthermore, CEA-specific T cell proliferation responses and protection against syngeneic, CEA-expressing adenocarcinoma cells were not observed among mice receiving AdCEA transfected dendritic cells. We conclude that this method of immunization is of low efficiency perhaps due to the low transfection frequency achieved with recombinant adenovirus in freshly isolated splenic or peripheral blood dendritic cells.

As a means to facilitate antigen uptake and presentation by dendritic Langerhans cells *in vivo*, we have examined co-delivery of GM-CSF cDNA in the context of polynucleotide

immunization by cutaneous particle bombardment. Indeed, co-delivery of GM-CSF cDNA in this context significantly augments the humoral and cellular immune response to cutaneous polynucleotide immunization by gene gun. This strategy was also examined as a means of augmenting the immune response to intramuscular polynucleotide immunization with no significant enhancement observed. However, based upon the immune augmentation observed with co-delivery of GM-CSF cDNA via gene gun, we have embarked upon the evaluation of a number of selected cytokine, growth factor, and co-stimulatory molecule cDNA's to augment the immune response to polynucleotide immunization via gene gun or i.m. delivery. In this regard, we have demonstrated that co-delivery of B7.1 cDNA augments the immune response and antitumor effects of polynucleotide immunization by i.m. delivery.[19] Our future efforts will be directed toward targeting of antigens to dendritic cells *in vivo*.

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Selected strategies to augment polynucleotide immunization

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We sought to amplify the immune response to polynucleotide immunization through co-delivery of complementary DNA (cDNA) encoding a cytokine or co-stimulatory molecule to enhance antigen presentation. In the context of intramuscular immunization, we examined co-delivery of cDNAs for B7–1 and human carcinoembryonic antigen (CEA) within separate plasmids or a dual plasmid with two independent expression cassettes. Intramuscular delivery of the dual expression plasmid produced anti-CEA antibody responses and antitumor effects superior to those generated by plasmid DNA encoding CEA alone. However, co-delivery of cDNAs encoding B7–1 and CEA in the form of two separate plasmids produced no augmentation. The importance of single plasmid delivery suggests the effectiveness of this strategy is contingent upon co-expression of B7–1 and CEA within the same cell. The success of cutaneous polynucleotide immunization by particle bombardment is thought to derive largely from the presence of Langerhans cells within the skin. We hypothesized that co-delivery of plasmid DNA encoding granulocyte–macrophage colony stimulating factor (GM-CSF) by particle bombardment would enhance the antigen presenting capacity of Langerhans cells at the inoculation site similar to its effects in vitro. Augmentation of CEA-specific lymphoblastic transformation and antibody response was observed when plasmid GM-CSF (pGM-CSF) was administered 3 days prior to each dose of plasmid DNA encoding CEA. These strategies for augmentation of immune response to polynucleotide immunization should be applicable to a wide variety of antigenic targets including infectious agents and other tumor-associated antigens.

Keywords: polynucleotide immunization; genetic immunization; carcinoembryonic antigen; cancer; gene gun

Introduction

There is increasing interest in the *in vivo* delivery of plasmid DNA encoding a relevant antigen as a novel approach to vaccination, hereafter referred to as 'polynucleotide immunization'.[1–3] The technique of direct intramuscular injection of plasmid DNA encoding specific antigens has elicited humoral and cellular immune responses to a variety of infectious agents including influenza, hepatitis B, human immunodeficiency virus, and others.[1–3] An alternate approach to polynucleotide immunization described by Tang *et al*[4] involves bombardment of the skin with gold microprojectiles coated with plasmid DNA encoding a specific antigen. This particle bombardment strategy has elicited humoral immune responses to human growth hormone[4,5] and influenza hemagglutinin as well as protection against influenza virus challenge.[6]

To examine the ability of polynucleotide immunization to achieve specific antitumor immunity, we have constructed a plasmid DNA encoding the full-length complementary DNA (cDNA) for human carcinoembryonic antigen (CEA) under transcriptional regulatory control of

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the cytomegalovirus early promoter/enhancer.[7] This plasmid delivered by intramuscular (i.m.) injection can function as a polynucleotide vaccine to elicit CEA-specific humoral and cellular immune responses as well as protection against syngeneic, CEA-expressing colon carcinoma cells.[7–9] These effects were comparable to the immune response and immunoprotection achieved with a recombinant vaccinia virus encoding CEA.[7]

When considering possible strategies to achieve immune amplification, we utilized recent observations pertinent to antigen presenting cell (APC) mechanisms. If the i.m. route of immunization utilizes the myocyte as an APC, it would seem to be ill-equipped for this purpose when compared to 'professional' APCs like splenic dendritic cells or Langerhans cells. In particular, cell surface expression of accessory molecules has not been reported in the context of myocytes, and thus we hypothesized that provision of B7–1 surface expression might enhance induction of immune response in a manner analogous to that reported with B7–1 transduction of low immunogenicity tumor cells.[10–12]

In contrast, the success of cutaneous polynucleotide immunization by particle bombardment is thought to derive largely from the presence of Langerhans cells, highly potent antigen presenting cells, within the skin. Resting Langerhans cells are weak accessory cells for the sensitization phase of primary T cell dependent immune responses.[13,14] However, Langerhans cells can mature

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immunologically in bulk epidermal culture and acquire most of the features of lymphoid dendritic cells including increased levels of major histocompatibility complex (MHC) class I and class II molecule expression. In this regard, granulocyte–macrophage colony stimulating factor has been identified as the principal mediator of immunologic maturation of Langerhans cells in culture.[13,14] Furthermore, GM-CSF plays an important role in determining the distribution and differentiated state of Langerhans cells in vivo.[15] Thus, we reasoned that cutaneous delivery of GM-CSF cDNA by particle bombardment with resultant local GM-CSF expression would induce immunologic maturation of Langerhans cells enhancing their ability to present co-delivered antigens. In this report, we have evaluated co-delivery of cDNAs encoding B7–1 or GM-CSF with cDNA encoding CEA as potential augmentation strategies for intramuscular or cutaneous polynucleotide immunization.

Results

Augmentation of intramuscular polynucleotide immunization by B7–1

To examine the effects of co-expression of human CEA and mouse B7–1 in striated muscle, three plasmids were utilized: (1) plasmid DNA encoding carcinoembryonic antigen (pGT37);[9] (2) plasmid DNA encoding mouse B7–1 (pGT59) shown in Figure 1a; and (3) plasmid DNA encoding mouse B7–1 and carcinoembryonic antigen (pGT64) as independent expression cassettes (Figure 1b). The ability of the B7–1 plasmids to direct expression of mouse B7–1 was confirmed by in vitro transfection of HeLa cells followed by fluorescence activated cell sorting (FACS) analysis for murine B7–1 expression and in situ hybridization to detect murine B7–1 mRNA. As shown in Figure 2, in situ hybridization clearly demonstrated murine B7–1 mRNA within many cells transfected with pGT59 (B7–1) and occasional cells transfected with pGT64 (CEA/B7–1). FACS analysis for cell surface expression of murine B7–1 protein directed by each plasmid is provided in Table 1. HeLa cells transfected with an irrelevant plasmid, plasmid DNA encoding no eukaryotic expression cassettes (pUC18), had fewer than 0.1% B7–1 positive cells while 74% of murine B7–1 stable transfectants were positive. Transfection with pGT59 (B7–1) or pGT64 (CEA/B7–1) produced 18% and 2.5% B7–1 expressing cells, respectively. Thus, both plasmids

directed murine B7–1 expression in vitro but pGT59 (B7–1) did so with approximately 10-fold greater efficiency.

To examine co-expression of B7–1 as a means to augment the immune response elicited by i.m. polynucleotide immunization, groups of five mice received a low dose (1 μg) of pGT37 (CEA) previously shown to produce little or no immune response to CEA; an equimolar dose (1.3 μg) of pGT64 (CEA/B7–1); or 1 μg of pGT37 (CEA) mixed with 50 μg of pGT59 (B7–1) by i.m. injection on days 1, 29 and 43. Two weeks after the last immunization, mice were killed to obtain sera for assay of immune response.

The effect of B7–1 co-expression on the immune response elicited by intramuscular CEA-polynucleotide immunization is shown in Table 2. All five mice receiving the dual expression plasmid were clearly positive for anti-CEA antibody with amounts of antibody comparable to animals immunized with optimal doses of vaccine, ie 100 μg of pGT37 (CEA) weekly for 4 weeks.[9] Delivery of cDNAs encoding CEA and B7–1 in separate plasmids (pGT37 and pGT59) produced modest antibody responses in only two of five mice while this low dose of pGT37 (CEA) alone produced no humoral immune response. Thus, co-delivery of B7–1 cDNA appears to augment the antibody response to CEA-polynucleotide immunization, particularly when the cDNAs for B7–1 and CEA are delivered within a single plasmid.

The ability of B7–1 co-expression to augment in vivo antitumor effects was characterized in groups of seven mice who received a single i.m. injection of 1 μg of pGT37 (CEA), an equimolar single dose of pGT64 (CEA/B7–1), or 1 μg of pGT37 (CEA) mixed with 50 μg of pGT59 (B7–1). All mice were challenged with 2×10^5 syngeneic, CEA-expressing adenocarcinoma cells 3 weeks after immunization with tumor growth plotted in Figure 3. All 14 mice receiving pGT37 (CEA) alone or with pGT59 (B7–1) developed rapidly growing tumors. In contrast, mice receiving the dual expression plasmid pGT64 (CEA/B7–1) have been followed for 70 days with only two of seven developing a tumor. One of the tumors in the pGT64 (CEA/B7–1) group grew more slowly than those observed in the other two groups. Thus, delivery of cDNAs encoding B7–1 and CEA within the same plasmid appears to augment the antitumor effects of low-dose CEA-polynucleotide immunization. In contrast, delivery of B7–1 cDNA on a separate plasmid has no detectable effect on tumor immunoprotection.
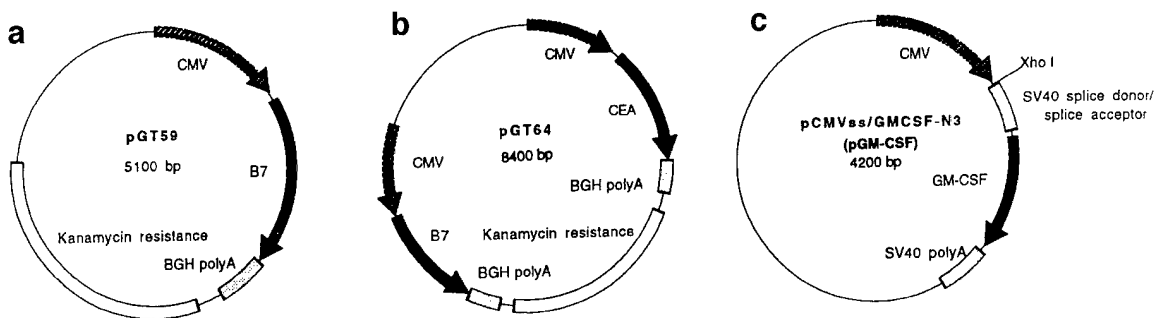

Figure 1 Expression plasmids. (a) pGT59 encoding murine B7–1 cDNA utilizing the pGT36 vector; (b) pGT64 encoding cDNAs for murine B7–1 and human CEA within two independent expression cassettes utilizing the pGT36 vector; and (c) pGM-CSF encoding murine GM-CSF cDNA utilizing the pNAss vector.
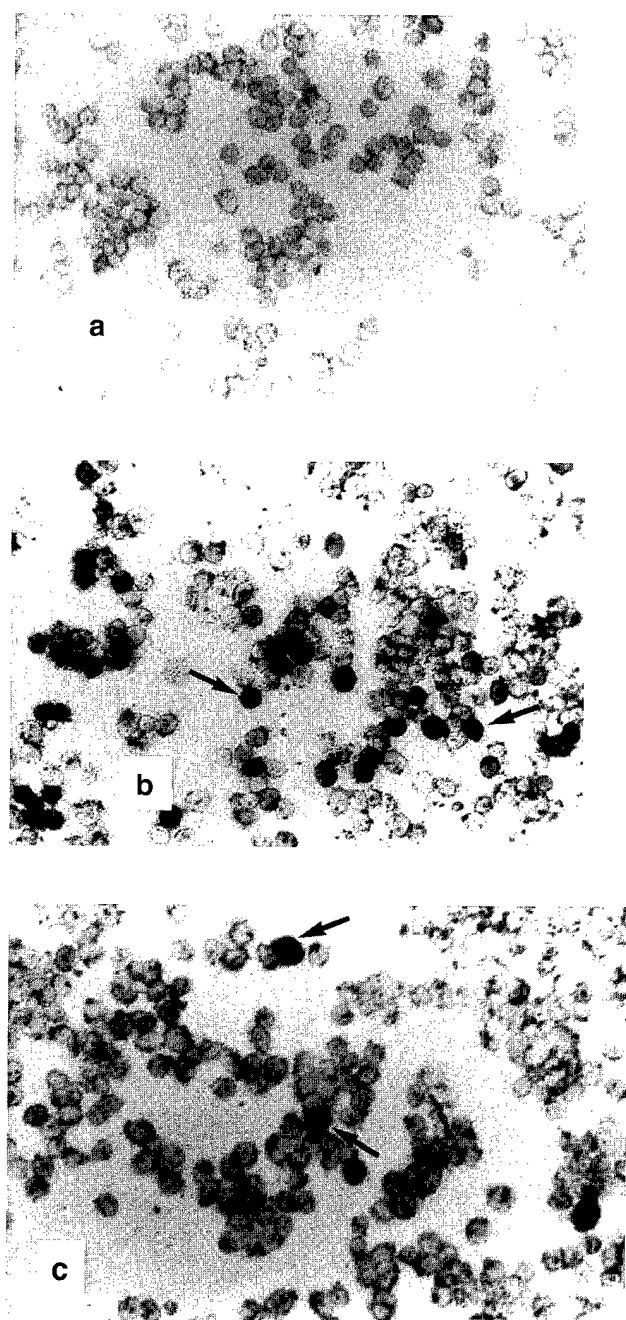
Figure 2 In situ hybridization for murine B7–1 in HeLa cells. (a) No B7–1 mRNA was evident among control cells transduced with irrelevant plasmid DNA, pUC18. (b) Transduction with pGT59 encoding B7–1 cDNA produced dark staining indicative of B7–1 mRNA expression in approximately 20% of cells. (c) Transduction with pGT64 encoding cDNAs for B7–1 and CEA produced B7–1 expression within approximately 3% of cells. Arrows denote cells staining positively for B7–1 mRNA.

Augmentation of cutaneous polynucleotide immunization by GM-CSF

The ability of GM-CSF co-expression to enhance the humoral and cellular immune response elicited by CEA-polynucleotide immunization via particle bombardment was evaluated using 2 μg doses of pGT37 (CEA). Groups of five mice received 2 μg of pGM-CSF either mixed with each dose of pGT37 (CEA) or delivered by particle bom-

bardment 3 days before each dose of pGT37 (CEA) at the same site. Control groups received pGT37 (CEA) alone or 2 μg of chloramphenicol acetyltransferase (pCAT) 3 days before each dose of pGT37 (CEA). Mice were immunized on days 1 and 29 with sera and spleens obtained 2 weeks after the last immunization for anti-CEA antibody and lymphoblastic transformation assays. Table 3 provides the anti-CEA antibody response with all five mice receiving pGT37 (CEA) alone demonstrating an anti-CEA antibody response whereas delivery of pGM-CSF 3 days before each dose of pGT37 (CEA) elicited anti-CEA antibody in all five mice with a mean level twice that of mice receiving pGT37 (CEA) alone. A one-way analysis of variance applied to ranks revealed that pretreatment with pGM-CSF significantly enhanced the antibody response compared to pGT37 (CEA) alone or pretreatment with plasmid DNA encoding pCAT ($P =$ 0.04 and $P < 0.01$, respectively). Concurrent administration of pGM-CSF diminished the immune response with only one of five mice positive for anti-CEA antibody ($P < 0.01$). Delivery of an irrelevant plasmid (pCAT) 3 days before each dose of pGT37 (CEA) to control for non-specific injury related to particle bombardment produced no enhancement of antibody response to CEA.

The same experimental groups of mice were analyzed with regard to splenic lymphoblastic transformation. CEA-specific lymphoblastic transformation data from the five mice receiving pGM-CSF 3 days before each dose of pGT37 (CEA) are given in Table 4. All five mice demonstrated dose-dependent lymphocyte proliferative responses to human CEA with peak stimulation ratios ranging from 34 to 692. All mice failed to respond to ovalbumin included as a control antigen with stimulation ratios ranging from 0.7 to 1.2, and mitogen responses were intact. Complete data from these five mice have been provided as mean c.p.m. with the standard error to illustrate the reliability of these assays as well as the dose-dependent nature of the CEA-specific immune responses in the context of appropriate positive and negative controls. We have never seen positive cellular immune responses to CEA in unimmunized animals or animals immunized with control plasmids.[7–9] All particle bombardment groups of mice were assayed with the same panel of antigens and mitogens over the same range of concentrations. To facilitate comparison of data between groups of mice, the results have been provided as stimulation ratios for cells stimulated with 10 μg/ml of CEA (Table 5). Three of five mice receiving the CEA plasmid (pGT37) alone demonstrated moderate CEA-specific lymphoblastic transformation whereas administration of pGM-CSF 3 days before pGT37 (CEA) elicited marked CEA-specific lymphocyte proliferative responses in five of five mice. A one-way analysis of variance applied to ranks demonstrated that this difference was significant ($P = 0.001$). Concurrent delivery of pGM-CSF diminished the immune response producing no evidence of lymphoblastic transformation ($P = 0.02$). Pretreatment with pCAT produced no enhancement of lymphocyte response to CEA. In all instances, response to control ovalbumin was negative and mitogen responses were brisk. Thus, pGM-CSF delivered by particle bombardment 3 days before each dose of pGT37 (CEA) produces substantial enhancement of the cellular immune response and modest enhancement of the humoral immune response to CEA. This effect is dependent upon the GM-

Table 1 B7–1 expression by fluorescence activated cell sorting for CTLA4-Ig binding

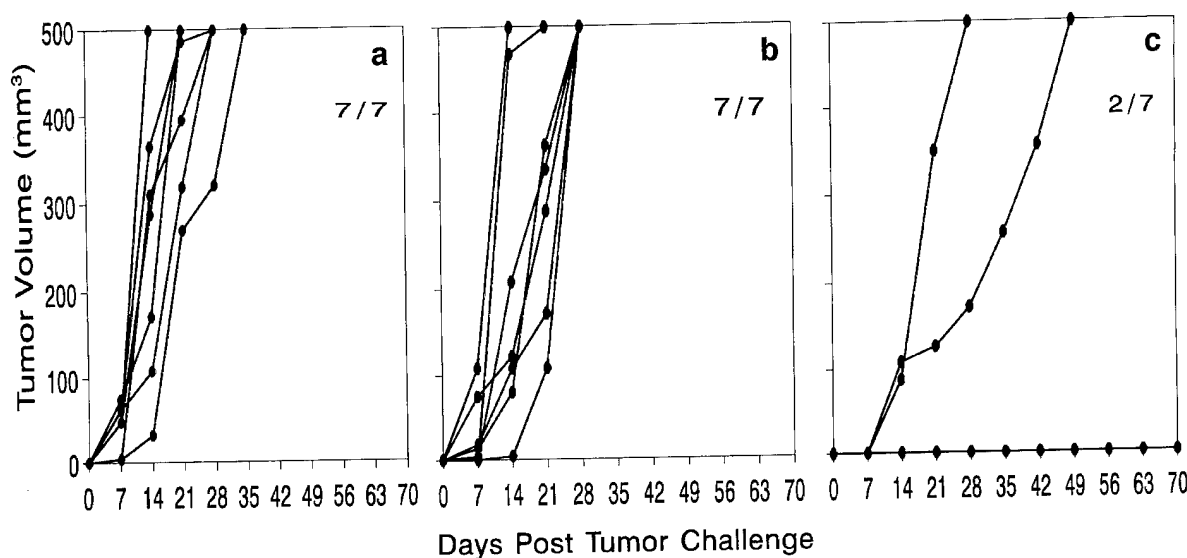| HeLa cell transfectants	No. of cells counted	No. of cells staining for B7–1	% of cells staining for B7–1
pUC18 (irrelevant plasmid)	3000	1	0.03
B7–1 stable transfectant	2992	2199	74
pGT59 (B7–1)	2996	529	18
pGT64 (B7–1/CEA)	3000	74	2.5

Figure 3 *Effect of intramuscular CEA polynucleotide immunization on growth of a transplanted mouse adenocarcinoma cell line expressing human CEA. Groups of seven mice received a single i.m. injection of 1 μg of pGT37 (CEA) shown in (a); 1 μg of pGT37 (CEA) mixed with 50 μg of pGT59 (B7–1) shown in (b); or 1 μg of pGT64 (CEA/B7–1) shown in (c). All mice were challenged with 2 × 10⁵ MC38-CEA-2 cells 3 weeks after immunization. Serial tumor measurements and the total number of tumor bearing mice over 70 days of observation are depicted.*

Table 2 Effect of B7–1 co-expression on the anti-CEA antibody response to intramuscular polynucleotide immunization[a]

Vaccine	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
pGT37 (CEA)	1[b]	0	4	0	0
pGT37 (CEA)/ pGT59 (B7–1)	0	0	0	47	87
pGT64 (CEA/B7–1)	133	689	423	262	98

[a]Groups of five mice received 1 μg of pGT37 (CEA), 1.3 μg of pGT64 (CEA/B7–1) or 1 μg of pGT37 (CEA) mixed with 50 μg of pGT59 (B7–1) by i.m. injection on days 1, 29 and 43 with sera obtained 2 weeks after the last immunization.
[b]Results expressed as ng of ¹²⁵I-CEA bound/ml of sera (see Materials and methods). A positive result is defined as exceeding 2 s.d. above the mean of normal mouse sera and is > 15 ng/ml.

Table 3 Effect of pGM-CSF co-expression on the anti-CEA antibody response to polynucleotide immunization by particle bombardment[a]

pGM-CSF administration	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
None	59[b]	70	93	60	109
pGM-CSF days 1 and 29	36	0	0	0	0
pGM-CSF days –2 and 26	183	216	65	159	149
pCAT days –2 and 26	0	50	106	115	0

[a]Groups of five mice received 2 μg of pGM-CSF either mixed with each dose of pGT37 (CEA) or delivered by particle bombardment 3 days before each dose of pGT37 (CEA). Control mice received pGT37 (CEA) alone or 2 μg of pCAT 3 days before each dose of pGT37 (CEA). All mice received 2 μg doses of pGT37 (CEA) on days 1 and 29 with sera obtained 2 weeks after the last immunization.
[b]Results expressed as ng of ¹²⁵I CEA bound/ml of sera (see Materials and methods). A positive result is defined as exceeding 2 s.d. above the mean of normal mouse sera and is > 15 ng/ml.

CSF cDNA since pretreatment with pCAT did not augment the immune response to CEA.

Discussion

Augmentation by B7–1
The field of polynucleotide vaccines and immunization strategies is approaching clinical application for both

Table 4 CEA-specific lymphoblastic transformation elicited by particle bombardment with pGM-CSF 3 days before pGT37 (CEA)[a]

	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
Control	18 ± 3[b]	20 ± 5	14 ± 3	15 ± 3	13 ± 2
CEA 10 µg/ml	620 ± 100	3000 ± 500	4700 ± 900	4400 ± 1000	9000 ± 3000
CEA 3 µg/ml	290 ± 50	2600 ± 500	3900 ± 1000	790 ± 300	6700 ± 600
CEA 1 µg/ml	160 ± 50	140 ± 50	88 ± 10	120 ± 40	2100 ± 800
OVA 100 µg/ml	14 ± 2	14 ± 2	14 ± 3	13 ± 3	16 ± 2
Con A	$14\,000 \pm 200$	$12\,000 \pm 200$	8800 ± 40	7900 ± 200	$33\,000 \pm 900$

[a]Mice (five) received 2 µg of pGM-CSF by particle bombardment 3 days before each 2 µg dose of pGT37 (CEA) on days 1 and 29 with splenic T cells obtained 2 weeks after the last immunization.
[b]Results are the mean c.p.m.s of quadruplicate wells ± s.e.m.

Table 5 Effect of pGM-CSF on the CEA-specific lymphoblastic transformation response to polynucleotide immunization by particle bombardment[a]

pGM-CSF administration	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
None pGM-CSF	9[b]	17	1	1	12
days 1 and 29 pGM-CSF	1	1	1	1	1
days –2 and 26 pCAT	34	150	336	294	689
days –2 and 26	2	1	1	1	2

[a]Groups of five mice were immunized with 2 µg doses of pGT37 (CEA) on days 1 and 29 with or without co-delivery of pGM-CSF or pCAT. Splenic T cells were obtained 2 weeks after the last immunization.
[b]Results are the mean stimulation ratio of quadruplicate wells stimulated with 10 µg/ml of baculovirus recombinant human CEA. A positive response is defined as a stimulation ratio ≥ 2.

infectious diseases and cancer.[9,16–18] Experience has shown that translation of murine models to man is often more difficult than expected and some observations suggest that muscle expression of plasmid DNA may be less efficient in primates than rodents.[19] With these observations in mind, our group has begun to explore general strategies to enhance the efficacy of polynucleotide immunization. This report notably demonstrates that co-delivery of cytokine or co-stimulatory molecule cDNAs can enhance polynucleotide induced immune responses and that immune responses to a tumor-associated antigen can be induced using a cutaneous polynucleotide particle bombardment system. Our studies using i.m. polynucleotide immunization demonstrated that co-delivery of B7–1 cDNA within a dual expression plasmid encoding CEA produced anti-CEA antibody responses and anti-tumor effects which were superior to those generated by plasmid DNA encoding CEA alone. However, co-delivery of B7–1 cDNA within a separate plasmid failed to enhance the immune response to plasmid DNA encoding CEA. The dual expression plasmid proved to be a more effective means of B7–1 cDNA co-delivery despite 10-fold lower in vitro transduction efficiency compared to separate plasmids encoding B7–1 and CEA. The importance of single plasmid delivery suggests that the ability of this strategy to enhance the immune response to CEA is con-

tingent upon co-expression of B7–1 and CEA within the same cell. This result is in accord with our understanding of B7–1 as a ligand present upon antigen presenting cells which interacts with the T cell co-stimulatory molecules CD28 and CTLA-4 during induction of the immune response to many antigens.[20,21] For B7–1 expression to be effective, it must occur on the surface of cells which are also expressing CEA immunogenic peptides in the context of MHC class I and class II molecules for presentation to T cells. The ability of B7–1 cDNA to enhance the immune response elicited by polynucleotide immunization provides indirect evidence that myocytes may function as antigen presenting cells as suggested by previous authors.[22–24] If the myofiber cells were simply providing a source of CEA protein to the draining lymph nodes or other distant sites where antigen presentation occurred, then expression of B7–1 by myofiber cells would be expected to have no effect on the immune response. The mechanism of antigen presentation in polynucleotide immunization is an important and active area of investigation. Further studies will be necessary to clarify the cells and molecular mechanisms involved.

Augmentation by GM-CSF

Cutaneous particle bombardment utilizing gold microbeads coated with plasmid DNA is an alternate delivery mechanism for polynucleotide immunization. The efficacy of this approach is thought to derive in part from delivery of antigen to epidermal Langerhans cells, potent antigen presenting cells within the dendritic cell family.[5] Prior studies have demonstrated humoral immune responses to viral antigens as well as protection against viral challenge in a variety of species.[4–6] This strategy has previously not been analyzed regarding generation of an immune response to a tumor-associated antigen.

We evaluated the capacity of GM-CSF cDNA co-delivery to augment the CEA-specific immune response elicited by cutaneous particle bombardment with plasmid DNA encoding CEA (pGT37). The GM-CSF cytokine was selected for use in this system based upon evidence that GM-CSF is the principle mediator of immunologic maturation of epidermal Langerhans cells in vitro[13,14] and plays an important role in determining the distribution and differentiated state of Langerhans cells in vivo.[15] Furthermore, Dranoff et al[25] demonstrated that transduction of tumor cell vaccines with GM-CSF cDNA could enhance the induction of potent antitumor immunity to poorly immunogenic tumors. We examined delivery of pGM-

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CSF 3 days before each dose of plasmid DNA encoding CEA *vs* concurrent delivery of both plasmids based upon evidence that protein expression by gene gun delivery and immune effects on Langerhans cells would require some time to occur. Augmentation of CEA-specific lymphoblastic transformation and antibody response was observed when pGM-CSF administration antedated CEA plasmid delivery while concurrent delivery of both plasmids prevented the CEA-specific immune response from occurring. The inhibition of immune response might be related to competition between the two genes for expression. Also, gene expression following particle bombardment is transient with most of the expression being lost within 3 days due to normal sloughing of epidermal keratinocytes.[5,26] Thus, simultaneous delivery of pGM-CSF and the CEA plasmid would not be expected to produce immunologic maturation of the Langerhans cells until the majority of CEA expression by keratinocytes was lost. Enhancement of the immune response to plasmid DNA encoding CEA by pretreatment with pGM-CSF appears dependent upon the GM-CSF cDNA rather than nonspecific effects related to the trauma of particle bombardment as pretreatment with pCAT produced no augmentation.

Thus, the immune response and antitumor effects elicited by i.m. polynucleotide immunization can be augmented by co-delivery of B7–1 cDNA within a dual expression plasmid, perhaps through enhanced antigen presentation by myocytes. Similarly, pretreatment with plasmid DNA encoding GM-CSF augments the immune response to cutaneous polynucleotide immunization by particle bombardment, perhaps through local immunologic maturation of Langerhans cells. The strategy of augmenting the immune response to intramuscular or cutaneous polynucleotide immunization by co-delivery of accessory molecule cDNAs is applicable to a broad range of vaccine applications including infectious agents and other tumor-associated antigens. Studies are ongoing to evaluate other cytokines, growth factors and co-stimulatory molecules as well as the molecular configuration and timecourse for their delivery.

Materials and methods

Plasmid DNAs for vaccination

Plasmid constructs: The gene for full-length human CEA[27] was used to construct an expression plasmid from the vector pCDNA3 (Invitrogen, San Diego, CA, USA), in which transcription is driven by the cytomegalovirus early promoter/enhancer.[7–9] For this construction, the neomycin resistance gene, the ampicillin resistance gene, and nonessential viral sequences were deleted from pCDNA3. The Tn903 kanamycin resistance gene from pUC4K (Pharmacia, Piscataway, NJ, USA) was inserted to allow selective propagation in *E. coli*. This expression vector is called pGT36.[9] The human CEA cDNA was then cloned into this modified eukaryotic expression vector to produce pGT37 (CEA) as previously described.[9] As control plasmids, we utilized pCDNA-CAT (Invitrogen) which contains the chloramphenicol acetyltransferase reporter gene in the pCDNA3 vector (designated pCAT) and pUC18 (GIBCO BRL, Grand Island, NY, USA), a procaryotic cloning vector containing no eukaryotic expression cassettes.

The cDNA for murine B7–1 was provided by the Genetics Institute (Cambridge, MA, USA). An *XbaI–Hin*dIII fragment containing the entire B7–1 open reading frame was excised and termini blunted via a Klenow fill-in reaction. This 0.9-kb fragment was cloned into the pGT36 expression vector described above.[9] The resulting murine B7–1 expression plasmid shown in Figure 1a is called pGT59. To achieve dual expression of human CEA and murine B7–1, a double expression vector was constructed. This derivative involved placement of two independent expression cassettes within the same plasmid backbone, in this case pGT36. Thus, a fragment encoding the murine B7–1 cDNA flanked by the cytomegalovirus (CMV) promoter/enhancer and synthetic poly(A) sequences was excised from pGT59 (B7–1) and cloned downstream of the CEA expression cassette in pGT37 (CEA) described above.[9] The resulting plasmid, pGT64 (CEA/B7–1), contains the two independent expression cassettes, whereby transcription within the two units is driven in opposite directions (Figure 1b).

Murine GM-CSF cDNA was provided by Dr Nicholas Gough[28] (WEHI, Melbourne, Australia). To enhance gene expression, a fragment corresponding to base pairs 131–620 of the murine GM-CSF cDNA was amplified via polymerase chain reaction. This fragment, representing the coding region without the 5′ and 3′ untranslated regions, was verified by DNA sequencing and subcloned into a pNAss vector (Clontech, Palo Alto, CA, USA) with the human cytomegalovirus promoter to give pGM-CSF (Figure 1c).

Plasmid purification: *E. coli* strain DH5α (GIBCO BRL) carrying the pGT37, pGT59, pGT64, or pGM-CSF plasmids was grown in Terrific Broth (GIBCO BRL). Antibiotic selection employed 50 μg/ml of kanamycin for pGT37 (CEA), pGT59 (B7–1) and pGT64 (CEA/B7–1) whereas 100 μg/ml of ampicillin was used for pGM-CSF. Plasmids were purified using Qiagen Plasmid Mega Kits (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions, with one Qiagen tip 2500 used for each liter of *E. coli* culture. Endotoxin was removed by extraction with Triton X-114 (Sigma, St Louis, MO, USA) as previously described.[9] The DNA was precipitated in large lots (5 mg) and stored at −70°C as pellets. For experimental use, the DNA was resuspended in sterile saline at a concentration of 5 mg/ml and stored in aliquots at −20°C for use in immunization protocols.

Functional validation of plasmid DNA

The ability of plasmids pGT59 (B7–1) and pGT64 (CEA/B7–1) to direct expression of murine B7–1 was studied before employment in immunologic studies. For this analysis, the plasmid DNAs pGT59 (B7–1) and pGT64 (CEA/B7–1) were transfected into the human cervical carcinoma cell line, HeLa, employing the adenovirus-polylysine method as described.[29] Cell surface expression of murine B7–1 was examined 24 h following transfection by FACS analysis using a fusion protein consisting of the extracellular domain of murine CTLA-4 and a human immunoglobulin Cγ chain kindly provided by Peter Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA, USA).[30] HeLa cells transfected with an irrelevant plasmid, pUC18 (GIBCO BRL), served as a negative control whereas RT11 mouse fibroblasts stably transfected with murine B7–1 cDNA, and

maintained under antibiotic selection, provided a positive control for murine B7–1 expression.

HeLa cells transfected as above were also examined for murine B7–1 mRNA expression using a hapenated single-stranded RNA probe (riboprobe) with an immunochemical detection system as previously described.[31] Briefly, the probe consists of antisense single-stranded RNA molecules incorporating digoxigenin-UTP.[31] Riboprobe bound to cellular murine B7–1 mRNA was detected by a specific sheep anti-digoxigenin F(ab)$_2$ antibody labeled with alkaline phosphatase.[31]

Verification of pGM-CSF expression was performed by transfection of murine B16 melanoma cells via gene gun particle bombardment. For this analysis, murine GM-CSF expression was quantified by ELISA assay (Pharmingen, San Diego, CA, USA) of cell culture supernatant obtained 18 h after transfection.

Cells and reagents
Production and maintenance of the human CEA transduced MC38-CEA-2 subline from the murine colonic adenocarcinoma cell line, MC38[32] has been previously described.[27] Before inoculation into animals, CEA expression was verified using murine monoclonal antibody COL-1 binding.[33] Native human CEA purified from hepatic metastases of human colonic adenocarcinoma was obtained from Vitro Diagnostics, Littleton, CO, USA.[34] Baculovirus recombinant human CEA was kindly provided by MicroGeneSys, Meriden, CT, USA. Ovalbumin, concanavalin A (con A), and spermidine were obtained from Sigma Chemical Company. Polystyrene beads (6.4 mm) were obtained from Precision Plastic Ball (Chicago, IL, USA). Gold beads (0.9 μm) were obtained from Degussa, South Plainfield, NJ, USA. Tefzel tubing was obtained from McMaster-Carr, Chicago, IL, USA.

Immunization method

Intramuscular injection: Six- to eight-week old female C57BL/6 mice (Charles River Laboratories, Raleigh, NC, USA) were anesthetized with ketamine and xylazine by intraperitoneal injection. Tongues were pulled out of the mouth gently with forceps to allow a 28-gauge needle to penetrate the bulk of the tongue muscle. All polynucleotide injections employed a constant 50 μl volume of normal saline with plasmid DNA concentration varying with dose. Preliminary studies by our laboratory[7–9] and others[35] have demonstrated that tongue injections of this volume do not impair the ability of animals to eat or drink following recovery from anesthesia. The tongue was selected to allow direct visualization of the striated muscle without necessitating a surgical procedure.

Cutaneous inoculation by particle bombardment: The preparation and immunization techniques for cutaneous immunization have been described previously.[5,6] In brief, 80 μg of either pGT37 (CEA), pCAT, pGM-CSF, or pGT37 (CEA) and pGM-CSF were added to a microcentrifuge tube containing 40 mg of 0.9 micron gold beads suspended in 100 mm spermidine. While gently vortexing the tube, 400 μl of 2.5 m CaCl$_2$ were added to precipitate the DNA on to the beads and the tube was allowed to stand for 10 min to complete the precipitation. The DNA coated beads (2 μg of DNA per mg of gold) were pelleted by a 10 s spin and the supernatants were removed. The

gold/DNA pellets were washed twice by vortexing in 1 ml ethanol, microcentrifuging 10 s, and removing supernatants. The gold/DNA beads were transferred to a 15-ml culture tube, resuspended in 5.7 ml of ethanol to give 7 mg of gold/DNA per ml of ethanol. Sonication for 10 s in a bath sonicator generated a uniform gold suspension. Using a syringe attached by an adapter, this suspension was drawn into a 76 cm length of Tefzel tubing, 1 ml (7 mg of gold/DNA) of suspension filling 18 cm of tubing, yielding 1 mg gold/DNA per 2.5 cm of tubing. The tubing was then transferred into a tube turner (Agracetus, Middleton, WI, USA). After allowing the gold beads to settle, the ethanol was slowly drawn off and the turner was rotated for 30 s, smearing the gold/DNA around the inside of the tubing. The residual ethanol was removed by passing nitrogen through the tubing for 3 min. The tubing was cut into 1/2 inch long tubes (equal to one immunization dose) and the tubes were loaded into the Accell gene delivery device. Six- to eight-week-old female C57BL/6 mice (Harlan/Sprague/Dawley, Indianapolis, IN, USA) were anesthetized as described above, and their abdomens were clipped. At adjacent sites on each abdomen, two doses of gold/DNA particles were delivered by a helium blast at a pressure of 400 pounds per square inch. Each site received 1 μg of DNA (1 μg for each vector in co-delivery) on ½ mg of gold.

Lymphoblastic transformation
This assay was performed as previously described.[7–9] Stimulated cells received baculovirus recombinant human CEA over a range of concentrations (1–10 μg/ml); ovalbumin (100 μg/ml) as a negative control antigen; or concanavalin A (con A) at 5 μg/ml as a positive control mitogen. The range of CEA concentrations described above provided optimal stimulation in our prior studies of pGT37 (CEA) immunized mice.[9] The stimulation ratio was calculated as mean cpm of the stimulated cells divided by mean c.p.m. of the control cells. A positive response was defined as a stimulation ratio $\geq$ 2.0.

Antibody assay
Antihuman CEA antibody was quantified using a double antigen immunoradiometric assay as previously described.[7,36] Briefly, polystyrene beads were coated with purified native human CEA (2 μg/bead) in phosphate-buffered saline (PBS), washed three times with PBS containing 1% bovine serum albumin (BSA) and stored in wash buffer at 4°C until use. Twenty microliters of mouse sera (normal control postvaccination) were diluted to 100 μl with PBS containing 1% BSA and incubated with a single coated bead (in duplicate) for 2 h on a laboratory oscillator at room temperature, washed with PBS and incubated with 100 μl of ^{125}I-labeled native human CEA (approximately 10^6 c.p.m. per μg) at 2 μg/ml for 1 h, rewashed with PBS and counted on a Micromedic automatic gamma counter. Background nonspecific binding of approximately 1% of the available ^{125}I CEA was subtracted from c.p.m. bound and the nanograms of CEA bound to the bead per ml of sera was calculated from the known specific activity of the ^{125}I CEA. A positive response (<15 ng/ml) has been defined as exceeding 2 s.d. above the mean value of 10 normal mouse sera.

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Tumor challenge

Mice were inoculated with 2×10^5 syngeneic MC38-CEA-2 cells by subcutaneous injection in sterile PBS through a 20-gauge needle over the flank as previously described.[8,9,34] Tumors were measured by caliper in two dimensions, and the volumes were calculated using the formula (width^2 × length)/2.[34] As few as 2×10^4 MC38-CEA-2 cells produce tumors in 70–100% of naive mice (unpublished observations).

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Polynucleotide-Mediated Immunization Therapy of Cancer

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The novel observations that intramuscular injection of plasmid DNA preparations could result in myocyte gene expression and induce immune responses to encoded immunogens has generated intense interest in this form of gene therapy. This phenomena can occur with both DNA and RNA reagents, and can be used in immune protection (vaccine) or therapy strategies. Immunization with DNA plasmids has generated protective immunity to a wide variety of pathogens and tumor cells in murine animal models. Immune response has occurred in a broad range of animal species following intramuscular injection of plasmid DNA encoding various immunogens as well as following other routes of administration (intravenous, intradermal, etc). The mechanisms responsible for induction of the immune response are as yet unclear, but responses include antibody production, T-cell proliferation, lymphokine release, generation of cytolytic T cells, and delayed hypersensitivity reactions. Plasmid DNA production and purification methods are relatively easy to standardize, and dual expressing plasmids allow incorporation of immune enhancement molecules or second immunogens. Plasmid DNA encoding nontransforming tumor-associated antigens are in development with a National Institutes of Health-approved protocol for carcinoembryonic antigen in colorectal cancer patients. Transforming tumor-associated antigens (eg, HER2/neu) may be approached with RNA or replicative RNA constructs for immunization. The efficacy of this immune approach will soon be examined in clinical trials in patients with cancer and the acquired immunodeficiency syndrome.
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THE FIELD of gene therapy has received considerable attention in the form of in vitro and animal model studies over the past decade. More recently, initial phase I clinical studies have begun, with a major emphasis on development of novel treatment strategies for cancer.[1] The majority of these cancer treatment trials involve attempts to induce or enhance patients' immune responses to their own tumor cells. This interest in tumor immunization has been fueled by major observations relevant to the field of tumor immunology. First, the cellular and molecular mechanisms involved in the induction of the immune response have been more fully delineated and provide insight into the shortcomings of tumor cells as antigen-presenting cells.[2] Second, the studies of Rosenberg's group using passive immunotherapy with interleukin-2 and antitumor lymphocytes have documented the feasibility of producing substantial tumor regressions in patients with metastatic hu-

man tumors.[3] Third, the use of human, immune T cells to identify their molecular antigenic targets has begun a catalogue of possible tumor antigens to serve as human "cancer regression antigens."[4-7] Finally, modern molecular genetic techniques have provided an array of potential immunization strategies, some of which are classified as gene therapy and others that use genetic constructs or products in active immunotherapy.[8]

Table 1 lists the three general strategies that underlie current tumor immunization paradigms. The first approach emphasizes that our knowledge of cancer regression antigens is quite limited and thus uses whole tumor cells that have been genetically modified in vitro or in vivo to enhance their immunogenicity.[9] The second approach emphasizes the advantage of having defined cancer regression antigens (eg, MART-1, gp100, etc) or tumor-associated antigens (eg, Her2/neu, carcinoembryonic antigen, or prostate-specific antigen) to focus the immune response and more readily allow the use of immune assays as intermediate markers.[10] The third strategy uses identified peptide epitopes of tumor antigens that are the MHC-transmitted recognition units for T-cell interaction as highly specific reagents for therapy and immune monitoring assays.[11,12]

This review will describe studies undertaken over the past 2 years as a collaboration between the Gene Therapy and Targeted Immunotherapy Groups of our cancer center. These studies have examined the feasibility of using polynucleotide-mediated immunization as a gene therapy strategy to induce immune responses and antitumor effects in preclinical animal model studies, leading to approval of an initial phase I trial of this strategy in patients with colon cancer.[13-15]

Table I. Current Active Tumor Immunization Strategies

Tumor cell immunization (eg, interleukin-2, interferon-γ, granulocyte-macrophage colony-stimulating factor transduced autologous tumor cells; in vivo transduction with vectors expressing foreign MHC molecules, lymphokines, or growth factors).

Tumor antigen immunization (eg, recombinant vectors expressing tumor antigens; polynucleotide-mediated immunization).

Immunogenic peptide immunization (eg, peptide epitopes derived from Her2/neu, mutated Ras, or mutated p53).

POLYNUCLEOTIDE-MEDIATED IMMUNIZATION

Although sporadic interest in DNA and RNA therapeutic strategies have occurred over many decades, the surprising observations of Wolff et al regarding skeletal muscle expression of intramuscular injected plasmid DNA[16] and of Yang et al on the use of DNA-coated gold bead projectiles to induce tissue expression of plasmid DNA[17] led to an explosion of interest in applying this technology to induce immune responses. This technology was initially labeled DNA vaccination, but its application to therapy of existing diseases (eg, the acquired immunodeficiency syndrome and cancer) and the capacity of both DNA and RNA to mediate effects have led to a more general terminology, ie, polynucleotide-mediated immunization (PMI). Wolff et al conducted a series of studies (reviewed in ref 18) to characterize the unusual phenomena that skeletal muscle cells have the ability to internalize plasmid DNA (and RNA) without the need for a vector or carrier. These terminally differentiated cells appear to use caveolae and T tubules for DNA uptake with subsequent gene expression from an episomal location without integration into the cell's DNA. Expression appears to occur for many months following a single injection (eg, luciferase), although expression of more immunogenic proteins may persist for only 7 to 10 days.[19] Myocyte lytic agents like bupivacaine or cardiotoxin are able to enhance gene expression, apparently due to enhanced DNA uptake by regenerating myocytes.[20-23] The polynucleotide reagent can be delivered to muscle by needle injection or jet injection systems.[24] An alternative strategy is to use DNA-coated gold particles as projectiles for bombardment of skin and other tissues.[17,25,26] When applied

to skin, gene expression can be seen in the epidermis and dermis over 4 to 7 days, reflecting cell turnover of this tissue.

Shortly after reports of gene expression in muscle or skin were published, it was found that antibody responses to the expressed protein could be demonstrated.[26-28] It is noteworthy that this methodology induces the host's cells to express the protein providing access to the MHC class 1 pathway as well as protein secretion providing access to the MHC class 2 pathway. Indeed, immune responses involving cytolytic T cells, helper T cells, delayed hypersensitivity reactions, and antibody response have all been noted. This technique of immunization has produced protective immunity against a broad range of infectious agents (eg, herpes,[29] influenza,[27] leishmaniasis,[30] malaria,[31] etc and enhanced allograft rejection[32] and protection against tumor cell challenge.[14,15] Most reports have used murine models, but PMI has been successful with a broad range of species, including rabbits,[24] ferrets,[33] dogs,[34] cattle,[29] chickens,[35] and monkeys.[36,37]

POLYNUCLEOTIDE-MEDIATED IMMUNIZATION TO CARCINOEMBRYONIC ANTIGEN

Our research group had embarked on a clinical trial of a recombinant vaccinia virus expressing human carcinoembryonic antigen (rV-CEA).[38] This strategy reflects paradigm 2 (Table 1) using a tumor-associated antigen. Since a pattern of preclinical studies using CEA as the target molecule had generated a Food and Drug Administration-approved clinical phase I trial of rV-CEA, we examined the capability of polynucleotide-mediated immunization to induce CEA-mediated antitumor immune effects. Carcinoembryonic antigen is a 180-kd membrane-anchored glycoprotein originally thought to be present only in adenocarcinomas and fetal gut, but which has subsequently been found in small amounts in normal adult colonic mucosa. The expression of CEA by adenocarcinoma cells is characteristic of human colonic, breast, and non–small cell lung cancer.[39,40] The small amount of this glycoprotein in normal tissue supports the view that it can be tolerated in humans. However, recent studies from three laboratories have documented the ability of patients with CEA-expressing tumors to mount an immune response to CEA following immunization. Foon et al reported the development of both humoral and

cellular immunity to CEA among patients receiving an anti-idiotype vaccine to CEA.[41] Tsang et al[38] reported the existence of cytolytic T-cell precursors to CEA following immunization of metastatic adenocarcinoma patients with rV-CEA, and our laboratory has demonstrated CEA-specific lymphoproliferation responses among Dukes' C colon cancer patients receiving rV-CEA.[42]

Similar to CEA, the vast majority of cloned tumor-associated antigens represent nonmutated, self-antigens present in some normal adult tissue (Table 2). Thus, issues of tolerance and potential autoimmune toxicity will be important considerations for this and almost any human tumor-relevant antigen. We selected CEA as a prototypic tumor-associated antigen for our initial studies of polynucleotide immunization, with plans to incorporate other tumor antigens in future studies.

Our initial report documented the ability of a plasmid DNA expressing the full-length cDNA for CEA (pCEA) delivered by intramuscular (IM) injection to elicit CEA-specific antibody and lymphoproliferative responses[13] as well as protection against challenge with syngeneic, CEA-expressing colon carcinoma cells.[14,15] These effects were comparable to the immune response and immunoprotection achieved with rV-CEA.[13]

DOSE AND SCHEDULE VARIABLES

The immune response and antitumor effects elicited by CEA polynucleotide immunization are dose and schedule dependent. We and other investigators studying a variety of antigens have observed that 50- to 100-μg doses of plasmid DNA administered IM produce optimal humoral and cellular immune responses to the encoded antigens in mice.[14,15,27,36] These data correlate with observations made by Manthorpe et al,[50] who examined luciferase gene expression in mice following IM injection of plasmid DNA. Increasing the dose of plasmid DNA from 10 to 50 μg resulted in a 35-fold increment in luciferase expression, whereas expression plateaued at doses above 50 μg. Interestingly, optimal immune responses have been observed with as little as 0.4- to 4-μg doses of plasmid DNA delivered by particle bombardment of skin via gene gun.[28,51] Similarly, we have demonstrated that 4-μg doses of pCEA administered by IM injection are sufficient to elicit CEA-specific antibody and lymphoproliferative responses as well as to protect against challenge with syngeneic, CEA-expressing colon carcinoma cells.[52] Our observations correlate well with those of Ulmer et al, who demonstrated that as little as 1-μg doses of influenza hemagglutinin or nucleoprotein DNA vaccines generate hemagglutination-inhibiting antibodies and cytolytic T cells, respectively, with a direct correlation between the amount of DNA injected and the magnitude of the antibody response up to 100-μg doses.[37] Thus, IM injection of low doses (1 to 4 μg) of plasmid DNA reported to be optimal for gene gun delivery elicits protective immune responses against tumor cells and various infectious agents, but optimal immune responses are observed with 50- to 100-μg doses in mice.

The dose range of pCEA producing optimal immune responses with IM administration appears to be only modestly affected by the size of the animal injected. For example, we have elicited CEA-specific immune responses in 5-kg rabbits and 10-kg dogs using 50- and 150-μg doses of pCEA, respectively (Table 3). These doses are comparable to the optimal dose range in mice weighing 150- to 300-fold less. Our observations correlate well with those of other investigators who have reported successful polynucleotide immunization of ferrets,

	Expression		Human Immune
Antigen (Gene)	Tumor Types	Normal Adult Tissues	Response Sources
CEA	Colon, breast, lung, etc	Gastrointestinal tract	38,41,42
HER2/neu	Breast, ovary, colon, lung, etc	Breast, gastrointestinal tract	11,12,43,44
MART-1 = Melan-A	Melanoma	Skin, retina	5,6
MAGE-1	Melanoma, breast, small cell lung	Testes	45
Tyrosinase	Melanoma	Skin, retina	7,46
pg100	Melanoma	Skin, retina	47
MUC-1	Breast, ovary, pancreas	Breast, pancreas, gastrointestinal tract	48,49

Table 2. Human Immune Response to Tumor-Associated Self-Antigens

Table 3. Summary of Our Experience With Plasmid Carcinoembryonic Antigen Immunization by a Variety of Routes in a Variety of Species

| | Species | | | | | |
| | Mouse | | | Rabbit | Dog | |
Route of Administration	Antibody Response	Lymphoproliferation	Tumor Protection	Antibody Response	Antibody Response	Lymphoproliferation
Intramuscular (needle injection)	+	+	+	+	+	+
Intramuscular (Biojector; Bioject Inc, Portland, OR)	ND	ND	ND	+	+	+
Intradermal (needle injection)	+	+	+	+	+	ND
Intradermal (gene gun)	+	+	−	ND	ND	ND
Intravenous	+	+	+	+	ND	ND
Intraperitoneal	−	ND	ND	ND	ND	ND
Subcutaneous	−	ND	ND	ND	ND	ND

Symbols: +, evidence of immune responses or protection against tumor challenge; −, absence of immune responses or protection against tumor challenge.

Abbreviation: ND, not done.

calves, and non-human primates against a variety of viral pathogens with 100- to 500-μg doses of plasmid DNA.[33,34,36,37]

Determination of a logical immunization schedule for polynucleotide vaccines is a complex issue. Unlike protein vaccines in which the immunogen is degraded or cleared from the body within a few days, plasmid DNA has been shown to direct the expression of reporter genes in muscle for up to 19 months, the life span of a mouse.[19] Furthermore, Yankauckas et al have demonstrated that a single IM injection of plasmid DNA-encoding influenza nucleoprotein generates nucleoprotein-specific humoral and cytolytic T-cell responses that persist for 1 year.[53] Thus, polynucleotide immunization has the unique potential to establish an antigen depot within myocytes that may play an important role in maintenance of immunologic memory.[54,55] However, we have observed that mouse myocytes expressing CEA following CEA polynucleotide immunization appear to die within 10 days, perhaps related to immune-mediated attack (unpublished observations). Thus, in the context of CEA polynucleotide immunization, a long-term antigen depot within myocytes may not exist, providing a rationale for repetitive administration. We have found that four weekly administrations of pCEA reliably generates CEA-specific antibody and lymphoproliferative responses as well as protection against tumor challenge within 6 weeks of beginning immunization.[14,15] Dose administration every 3 weeks generates comparable immune responses and antitumor effects within 8 to 9 weeks.

ROUTE OF POLYNUCLEOTIDE IMMUNIZATION

Two predominant techniques for polynucleotide immunization have emerged in the literature: direct IM injection of plasmid DNA and delivery of plasmid DNA-coated gold particles to the epidermis by gene gun. To compare these two methods with regard to the nature of an immune response to a tumor-associated antigen, we delivered comparable doses of pCEA by IM injection or gene gun according to the same schedule.[52] Carcinoembryonic antigen-specific lymphoproliferative and anti-CEA antibody responses of comparable magnitude were observed following immunization by either technique. However, only IM injection provided immunoprotection against challenge with syngeneic, CEA-expressing colon carcinoma. The anti-CEA antibody response elicited by gene gun epidermal delivery was exclusively of Ig1 isotype, whereas IM injection produced both Ig1/Ig2a responses.[52] The latter isotype pattern is consistent with activation of T-helper 1 cells, which may be pivotal to the induction of antitumor effects. This differential effect may relate to the particular combination of mouse strain (C57BL/6) and antigen used for our experiments.

Other investigators have shown that injection of soluble plasmid DNA encoding influenza nucleoprotein into the superficial skin via needle and syringe produces discrete foci of epidermal and dermal cells expressing the encoded antigen, with resultant nucleoprotein-specific immune responses and pro-

tection against influenza viral challenge.[56] This suggests that intradermal injection of plasmid DNA may be an alternative method of polynucleotide immunization, although direct comparison with the gene gun delivery system has not yet been performed. We have shown that intradermal injection of pCEA elicits CEA-specific antibody and lymphoproliferative responses as well as protection against challenge with syngeneic, CEA-expressing colon carcinoma cells in mice (Table 3). In addition, intradermal injection of pCEA induced anti-CEA antibody responses in rabbits and dogs comparable to IM delivery (Table 3).

Intravenous (IV) injection represents another promising route for polynucleotide immunization. Robinson et al demonstrated humoral immune responses and protection against influenza viral challenge following IV injection of plasmid DNA encoding influenza hemagglutinin in mice and chickens.[35,57] In this regard, we have shown that IV injection of pCEA elicits CEA-specific antibody and lymphoproliferative responses as well as protection against tumor challenge in mice (Table 3). This technique also translates well to larger animal species with induction of anti-CEA antibody responses in rabbits comparable to IM injection.[58] The site of encoded antigen expression following IV injection of plasmid DNA remains uncertain. Other investigators examining the fate of plasmid DNA complexed with cationic lipids delivered IV in mice have reported that intact plasmid DNA is rapidly degraded in plasma with a half-life of less than 5 minutes.[59] Intact DNA was demonstrated in the lung, spleen, liver, heart, kidney, marrow, and muscle by Southern blot analysis and the polymerase chain reaction; however, immunohistochemical analysis did not detect encoded protein expression.[59] Kawabata et al recently showed that naked plasmid DNA is rapidly eliminated from the plasma following IV injection due to extensive uptake by reticuloendothelial cells in the liver.[60] Studies are under way at our institution using reverse-transcriptase polymerase chain reaction to define the sites of antigen expression following IV polynucleotide immunization.

With regard to IM polynucleotide immunization, other investigators have reported enhanced immune responses with use of the Biojector (Bioject Inc) needleless jet injection system.[24] The Biojector uses a CO_2 cartridge to deliver plasmid DNA in aqueous solution IM. It has been hypothesized that this strategy might result in a better distribution of plasmid DNA within the muscle. Our experience with pCEA has shown immune responses following Biojector administration that are comparable to those obtained with an ordinary needle and syringe in dogs[34] and rabbits (Table 3).

IMMUNE MECHANISMS IN POLYNUCLEOTIDE-MEDIATED IMMUNIZATION

The mechanisms responsible for induction of immune response by PMI have not been clearly delineated. Four hypotheses summarized in Table 4 could account for the elicitation of specific humoral and cellular immune responses. First, myocytes could function as antigen-presenting cells[61,62] with intracellular synthesis of antigen followed by MHC class I peptide display promoting T-cell activation. Cell surface expression or secretion of the antigen could provide B-cell activation. In support of this hypothesis, myocytes have been shown to constitutively express MHC class I molecules, and MHC class II expression is inducible with interferon-γ.[61,62] Furthermore, myoblasts treated with interferon-γ have been shown to present antigen to previously primed T cells with resultant cytotoxicity and/or T-cell proliferation.[63] Further support for this hypothesis derives from our observation that co-delivery of B7-1 cDNA within a dual expression plasmid encoding CEA produces anti-CEA immune responses and antitumor effects that are superior to those generated by the same amount of plasmid DNA encoding CEA alone or separate plasmids encoding CEA and B7-1.[64] The requirement for B7-1 cDNA co-delivery to muscle suggests that co-expression of B7-1 and CEA in myocytes enhances their antigen-presenting cell function. If myofiber cells were simply providing a source of CEA protein to "highly efficient" antigen-

Table 4. Mechanism of Polynucleotide-Mediated Immunization
Myocytes function as antigen-presenting cells.
Myocytes generate antigen, which travels to draining nodes for presentation.
Highly efficient antigen-presenting cells are recruited to the muscle by a nonspecific inflammatory response to plasmid DNA.
Plasmid DNA transfection of highly efficient antigen-presenting cells in draining nodes or spleen.

presenting cells, which characteristically express B7-1, then co-delivery of B7-1 cDNA would be expected to have no effect on the immune response. A second hypothesis calls for myocytes to simply provide an endogenous source of antigen to draining lymph nodes. There, antigen could be recognized by B cells as well as be processed by "highly efficient" antigen-presenting cells (dendritic cells, macrophages, and B cells) for presentation to T cells with appropriate co-stimulation. No experiments have explored this mechanism. A third hypothesis calls for plasmid DNA injection to elicit a nonspecific inflammatory response within the muscle that serves to recruit "professional" antigen-presenting cells to the injection site. These "professional" antigen-presenting cells then process and present antigen synthesized and released by injured myocytes. In support of this hypothesis, we have observed an inflammatory cell infiltrate along the needle track in muscle within 72 hours of plasmid DNA injection. Furthermore, other investigators[65] have shown that mammalian immune systems can recognize bacterial DNA because it contains unmethylated CpG dinucleotides resulting in potential adjuvant effects. Bacterial DNA is a known contaminant of plasmid DNA preparations grown in Escherischia coli and may be responsible for this early local inflammatory reaction. We have also demonstrated that combining 50 μg of irrelevant plasmid DNA-encoding chloramphenicol acetyltransferase with a low dose (1 μg) of pCEA for IM injection significantly augments the CEA-specific immune response, as illustrated in Fig 1. This observation is consistent with augmentation of CEA antigen presentation secondary to an enhanced local inflammatory response induced by irrelevant plasmid DNA. A fourth hypothesis calls for transfection of a small number of "professional" APCs by pCEA, which escapes the muscle and travels to draining lymph nodes or the spleen. The fact that intravenous routes of PMI can induce specific immune responses may support this alternative. Delineation of the mechanisms responsible for PMI will facilitate the rational design of strategies to enhance the efficacy of this immune therapy.

We have also evaluated the effector limb of the immune response by examining the phenotype and potency of splenocytes induced by CEA-polynucleotide immunization using the Winn assay.[66,67] For this purpose, mice received 50-μg doses of pCEA weekly for 6 weeks and were killed 1 week after the
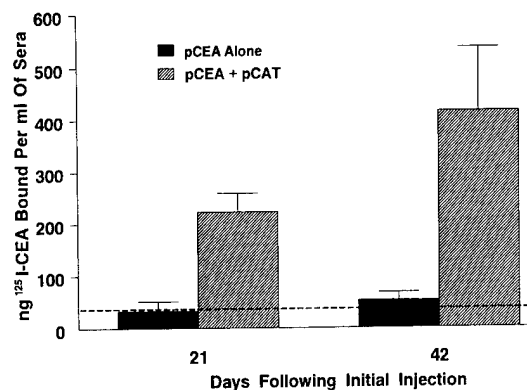

Fig 1. Augmentation of the anti-CEA antibody response to CEA polynucleotide immunization by co-delivery of irrelevant plasmid DNA. Groups of seven mice received low doses (1 μg) of plasmid DNA-encoding CEA (pCEA) by IM injection alone or mixed with 50-μg doses of irrelevant plasmid DNA-encoding chloramphenicol acetyltransferase (pCAT) for two injections 3 weeks apart. Sera for anti-CEA antibody response were obtained 21 and 42 days following the initial injection. Results are reported as nanograms of ^{125}I-CEA bound per milliliter of sera. The dashed line indicates the threshold for a positive result.

last injection to obtain nylon wool-enriched immune splenic T cells. Magnetic activated cell sorting was used to deplete either CD4+ or CD8+ T-cell subsets. Groups of five naive mice received 2 × 10⁵ syngeneic, CEA-expressing colon cancer cells mixed in various ratios with unfractionated immune T cells, CD4-depleted immune T cells, CD8-depleted immune T cells, or naive T cells and tumor growth measured. As illustrated in Fig 2, tumors grew in all mice receiving naive T cells, whereas immune T cells protected against tumor challenge at the remarkably low ratio of two
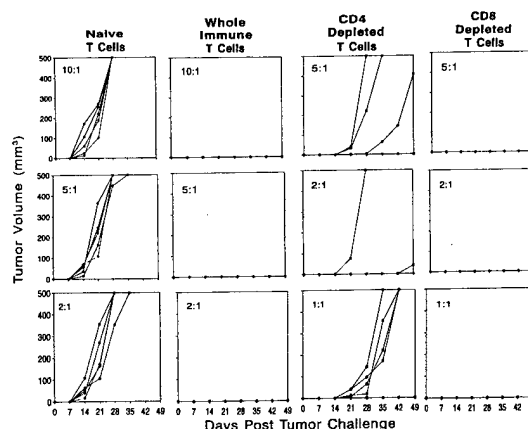

Fig 2. Phenotype and antitumor potency of splenocytes induced by CEA polynucleotide immunization as determined by the Winn assay.

effector T cells to one tumor cell. The CD8-depleted T-cell population was as protective against tumor growth as unfractionated T cells, while the CD4-depleted population had a reduced antitumor effect. To verify that the antitumor effect is mediated by T cells, immune splenocytes were positively selected for both CD4$^+$ and CD8$^+$ cells. This highly purified T-cell population completely eradicated tumor growth at a 1:1 ratio by the Winn assay (data not shown).

We have also examined the phenotype of antitumor effector cells through immunohistochemical analysis of the tumor transplantation site at various timepoints in naive and pCEA immunized mice. Mice immunized with pCEA develop extensive infiltration of the tumor by predominantly CD4$^+$ T cells (>300 T cells/mm^2) within 48 hours of challenge with syngeneic, CEA-expressing colon carcinoma cells, whereas naive mice demonstrate no T-cell infiltration at this timepoint. These data correlate well with the Winn reactions described above, which suggest that the predominant antitumor effector cells are CD4$^+$ T cells.

The antitumor effects that we have observed following CEA polynucleotide immunization could involve specific killing of individual cells based on their CEA expression, as expected with cytolytic T cells or nonspecific killing of tumor cells in bulk triggered by CEA within the milieu. The later

possibility includes such mechanisms as release of cytokines (eg, tumor necrosis factor), nonspecific phagocytosis, or disruption of neovasculature. To probe this question, we immunized groups of mice with pCEA and challenged them with 5 × 10^5 MC38-CEA cells or a mixture of 2.5 × 10^5 MC38-CEA cells and an equal number of MC38 cells without CEA expression, as illustrated in Fig 3. Immunization with pCEA protected nine of 10 mice challenged with MC38-CEA cells, with tumor outgrowth seen in seven of seven naive control mice. However, pCEA immunization failed to protect against challenge with a mixture of MC38 cells with and without CEA expression, with tumor outgrowth observed in 10 of 10 mice; the timecourse was similar to that seen in naive mice. Thus, the antitumor effector mechanism elicited by CEA polynucleotide immunization appears to be targeted at individual cells based on their CEA expression, with little evidence of innocent bystander effects. Further studies are ongoing to better clarify the effector mechanisms of these antitumor effects.

DNA CONSTRUCTS FOR POLYNUCLEOTIDE-MEDIATED IMMUNIZATION

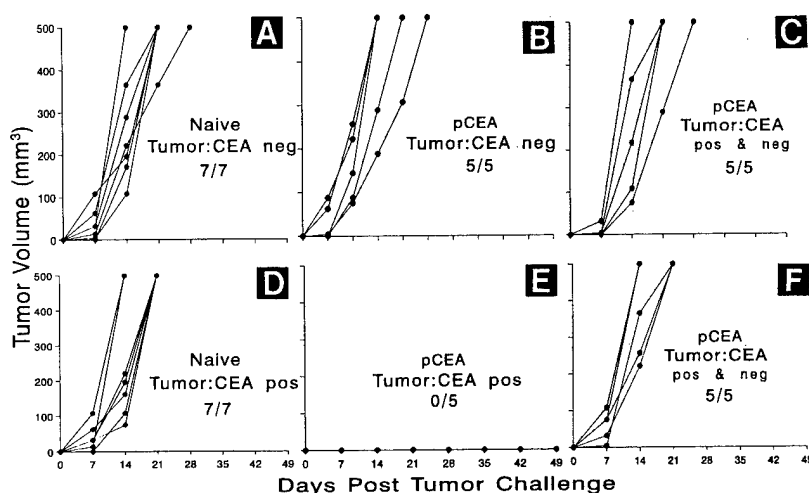In our original report,[13] expression of human CEA was achieved by incorporation of the cDNA

Fig 3. Carcinoembryonic antigen specificity of the antitumor effects elicited by CEA polynucleotide immunization. Groups of five mice received 50-μg doses of pCEA by IM injection weekly for 3 weeks followed by tumor challenge 1 week after the last injection. Groups of seven naive mice tumor challenged the same day served as controls. All mice received 5 × 10^5 tumor cells except those in panel F, which received 10^6 cells. Naive mice were challenged with CEA-negative (A) or CEA-positive (B) tumor cells. Mice immunized with pCEA were challenged with CEA-negative cells (C), CEA-positive cells (D), or an even mixture of CEA-positive and CEA-negative cells (E and F). Tumor growth curves as well as the number of mice displaying tumor growth over 7 weeks of follow-up are depicted.

into a commercially available eukaryotic expression vector. For human use application, however, specific elements of the vector backbone would be functionally extraneous or, more significantly, present potential safety hazards. To modify this plasmid for human use, we undertook specific modifications of the vector backbone.[15] The neomycin expression cassette was deleted, as these sequences were irrelevant to our proposed use in the human context. In addition, ampicillin selection may prove problematic due to delivery of trace amounts of ampicillin to penicillin-sensitive subjects. We thus replaced this selection system by incorporating a kanamycin resistance gene as a selective marker. The resulting plasmid, termed pCEA, is configured to contain the minimum elements essential for its propagation and utilization in the polynucleotide immunization context.[15]

In addition to the modified plasmid configuration, the DNA purification schema required amendment to be consistent with the anticipated human use of the product. Standard plasmid purification methods involve ethidium bromide dye with cesium chloride gradient separation. From the biosafety standpoint, the possible carryover of the powerful mutagen ethidium bromide represented an unacceptable risk. Alternative purification schemas were thus explored. Affinity chromatography matrix separation using a Qiagen column system was analyzed for its utility in this regard. This described methodology was also coupled to a lipid extraction procedure to remove contaminating endotoxin in the DNA preparation. It could be shown that use of the Qiagen purification system, in conjugation with the kanamycin selectable marker, allowed yields of plasmid comparable to standard techniques. In addition, analysis of the purity of the plasmid DNA showed it to be relatively free of protein and endotoxin contamination. Importantly, the bioactivity of the modified plasmid prepared in this manner was comparable to that observed in previous studies of CEA.[15]

A second major modification of our plasmid DNA construct design introduced the use of dual gene-expressing plasmids. As described previously, this allowed us to explore the effects of B7-1 expression following IM injection of a mixture of plasmids encoding CEA or B7-1 compared with a single plasmid construct encoding both CEA and B7-1. Co-delivery of distinct genes on separate plasmids would accomplish co-expression in the

same cell as a relatively rare event, whereas the dual expressing plasmid provides B7-1 expression in the same cell that expresses CEA. As described above, enhancement of CEA-specific immune responses and antitumor effects was observed with the dual expression plasmid but not with the use of separate plasmids encoding CEA and B7-1.[64]

A second purpose for the dual expressing plasmid design related to our proposed phase I trial in colon cancer patients. This initial trial will examine toxicity issues and immune response to CEA after IM injection of varying doses of our CEA-expressing plasmid DNA. This initial PMI trial will be performed in patients with metastatic colorectal cancer, who often have substantial tumor burden and elevated plasma levels of CEA. Failure to generate an immune response to CEA could thus reflect their clinical status or a shortcoming of the immunization reagent, dose, or schedule. The ability to co-express a highly immunogenic molecule like hepatitis B surface antigen (HBsAg) would allow an internal positive control for the efficacy of the immunization strategy. To accomplish this goal, plasmid strategies were derived to allow co-expression of CEA and the HBsAg. Plasmids were constructed whereby CEA and HBsAg were expressed via distinct expression cassettes, via a single expression cassette but linked by a viral internal ribosome entry site, or as a fusion gene expressed from a single promoter. Preliminary in vivo studies demonstrated the utility of the former approach in achieving co-expression of the two antigenic determinants. The plasmid pGT 63, which encoded CEA and HBsAg driven from separate CMV promoters, was thus used to immunize animals. This plasmid was capable of eliciting an immunologic response to both antigens. Our clinical trial approved by the Recombinant DNA Advisory Committee will therefore provide data related to immunity to a tumor-associated antigen as well as information related to the basic efficacy of this vaccine approach in humans.

RNA CONSTRUCTS FOR POLYNUCLEOTIDE-MEDIATED IMMUNIZATION

It should be noted that a subset of tumor-associated antigens are transformation-associated oncoproteins. Thus, in addition to the capacity to be recognized as immunogenic, they also participate directly in the process of neoplastic progres-

sion and conversion through their function as transforming agents. In this regard, immunity to the erbB-2 transmembrane receptor with tyrosine kinase activity has been demonstrated in a subset of patients with carcinoma of the breast or ovary.[11,12,43,44] In these tumor types, dysregulated expression of this growth factor receptor is also a key disease progression and prognostic factor. The delivery of plasmid DNA encoding the erbB-2 gene raises significant safety issues not relevant in the context of PMI for nontransforming tumor-associated antigens. These safety issues relate to the possible integration of the delivered erbB-2 cDNA into the host chromosome. Dysregulated expression of the incorporated gene could allow for malignant transformation of the genetically modified cell.[68-70] Specific strategies to achieve gene-based immunization against erbB-2 have been developed to circumvent the safety issues discussed above. It has been suggested that the immunogenic and transformation-associated domains of the erbB-2 molecule can be uncoupled using cDNA fragments encoding discrete portions of the mature erbB-2 molecule. This strategy may limit the potential for an integration event leading to cellular transformation. However, recombination between the delivered constructs encoding erbB-2 domains and endogenous erbB-2 could still conceivably allow dysregulated expression with the consequence of malignant transformation.

We have considered an alternative strategy using naked mRNA as a polynucleotide immunization vehicle. In this regard, mRNA would seem to offer certain intrinsic advantages. Foremost among these advantages is the recognition that the basic flow of genetic information would countermand any possibility of integration of the delivered genetic material into the host chromosome. The utility of mRNA as an expression vector was described by Malone et al, who used cationic liposomes to effect in vitro transfection of various target cells with mRNA transcripts derived via in vitro transcription reactions with the T7/SP6 viral polymerase system.[71] They showed that incorporation of a modified guanacil cap was key to the translation-competence of the derived synthetic transcript. In addition, the inclusion of heterologous 5' and 3' untranslated regions expropriated from the human β-globin gene allowed enhanced expression from the chimeric mRNA transcripts.

Based on these concepts, we developed a mRNA

approach to achieve antitumor immunization via PMI. Whereas this strategy was undertaken in the context of devising methods for anti-erbB-2 immunization, we initially used CEA as the target antigen for proof of principle studies. Based on the findings of Malone et al, we incorporated the 5' and 3' flanking regions of the human β-globin gene and transcription conditions were established for direct incorporation of modified guanacil capping of the derived transcripts. After in vitro synthesis, agarose gel electrophoresis confirmed the derivation of a transcript of the predicted size and configuration. It was thus feasible to derive large amounts of a homogenous species of CEA or luciferase-encoding mRNA. The CEA mRNA transcripts were transfected into CEA-negative cells via cationic liposome vectors. These transfected cells became CEA antigen positive, documenting their translation competence.[72] We next examined their ability to mediate protein expression in vivo following IM injection using luciferase transcripts to characterize the amount and duration of protein expression. For this analysis, direct IM delivery of 50 μg of either luciferase-encoding plasmid DNA or luciferase-encoding mRNA was accomplished in C57BL/6 mice. At various times postdelivery, transduced muscle groups were analyzed for expression of the luciferase reporter gene. Luciferase activity could be readily detected in the group of animals that received the luciferase-encoding mRNA, validating the functional utility of this vector approach. However, luciferase activity consequent to mRNA transduction peaked at 8 hours postinjection and returned to baseline within 3 days.[73] In contrast, levels of gene expression deriving from the plasmid DNA transcript were of a much greater magnitude and longer duration. This pattern of mRNA expression suggested a more intensified immunization schedule than used with plasmid DNA vectors.

In a preliminary study using 50 μg of CEA mRNA administered twice weekly for 5 weeks, mice were shown to be primed for an anti-CEA antibody response following challenge with CEA-expressing tumor cells.[72] This effect was suboptimal, presumably reflecting the one to two orders of magnitude greater protein expression mediated by plasmid DNA preparations.[73] To enhance RNA expression, consideration was given to the class of RNA vectors that can accomplish a self-replicative step after transduction. To this end, a series of RNA vectors were developed for transduction of

eukaryotic cells based on the Toga virus group. The viruses (poliovirus,[74] Semliki Forest virus,[75-77] Sindbis virus[73,78,79]) all possess single-strand RNA genomes. As part of their life cycle, the RNA genomes undergo self-replication prior to packaging and completion of the infection cycle. Recombinant RNA vectors had been derived from the viruses that retained the self-replicative capacity of the RNA genome. These transcripts, however, were not capable of inducing an infectious cycle as the viral genomes were devoid of the gene regions encoding viral packaging functions.[74,77,79] Thus, these single-strand RNA virus genomes were self-replicative but noninfectious. Furthermore, heterologous sequences could be incorporated into the viral genome in a context to allow expression of the encoded sequence in functional linkage with the self-replicative genome. Thus, the self-replicative mRNAs of the Toga virus family appeared to offer a means of amplifying expression following cell transduction.

The utility of the self-replicative mRNA vectors had been described principally in the context of eukaryotic protein expression. Vector vehicles constructed for this purpose include both packaged and nonpackaged mRNAs containing encoded transgenes.[76] The advantage of these systems was in the high levels of gene expression obtainable as well as the broad tissue tropism of the virions.[78,79] For application to PMI, we required that these vectors be capable of accomplishing in situ transduction of muscle cells after direct in vivo delivery. In addition, efficient self-replication would need to occur within striated muscle cells. We thus analyzed the utility of self-replicative RNA vectors as a strategy to achieve increased protein expression following IM injection.

As an initial approach, we used a vector system based on the Sindbis virus. We modified the TLXN Sindbis virus vector to contain a luciferase reporter gene. This plasmid (TLXN-Luc) thus contains the SP6 RNA polymerase promoter followed by Sindbis virus nonstructural genes required for RNA replication, a subgenomic promoter, the luciferase coding region, and a poly(A) sequence (72). Synthetic mRNA transcripts were derived from this plasmid by in vitro transcription. As a control, luciferase transcripts were also derived that lack self-replicative capacity. As an initial proof of functional utility, we examined the temporal pattern of reporter gene expression in BHK21 cells trans-

fected in vitro with the replicative or nonreplicative mRNA species. The replicative transcript accomplished up to two orders of magnitude higher levels of luciferase expression than the nonreplicative luciferase mRNA early (8 hours) and late (7 days) posttransfection.[72] These results confirm the findings of Xiong et al, who demonstrated that the self-replication capacity of the Sindbis virus genome allowed for extremely high levels of expression of encoded heterologous genes.[78] We then analyzed the potential utility of this approach in vivo by analyzing the ability of the Sindbis virus RNA transcripts to accomplish effective in situ transduction of muscle after direct injection. This analysis compared the nonreplicative and replicative luciferase encoding mRNAs following delivery of a 50-μg dose by IM injection. The replicative RNA generated higher and more prolonged levels of reporter gene expression than the nonreplicative reagent.[73] Analysis of replicative mRNA constructs encoding CEA and erbB-2 in terms of induction of immune responses are ongoing, but this strategy should allow extension of PMI to oncogenic protein applications.

ADVANTAGES OF POLYNUCLEOTIDE-MEDIATED IMMUNIZATION

This form of active immunotherapy has numerous advantages applicable to clinical trials. As tumor regression (or associated) antigens are identified, their cloned genes are readily incorporated into standard plasmid constructs. The generation and purification of plasmid DNA can be readily standardized and are applicable to a whole range of tumor antigen targets. The use of dual-expression vectors allows positive control antigen delivery to assess the functional integrity of the reagent, dose/schedule, and immune capacity variables. This strategy minimizes specificity variables in that the immunizing agent is a polynucleotide while immune monitoring assays use the protein antigen target. The specific immune assays can serve as intermediate markers in early clinical trials to establish optimal dose and schedule variables. We believe these characteristics represent important and practical issues in addressing human trials. However, it should be emphasized that observations on feasibility and efficacy of this strategy in humans are not yet available. An initial phase I trial of a dual-expressing plasmid DNA (CEA and

HBsAg) in patients with metastatic colorectal cancer has recently received approval by the Recombinant DNA Advisory Committee and National Institutes of Health director. Results from this proposed trial and other human trials of polynucleotide-mediated immunization will provide important observations on the relevance of this strategy to human disease.

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